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CORRIGENDUM

In Catlin, B. W. and Cunningham, L. S. (1958). *J. gen. Microbiol.* **19**, 522–539.

On p. 529, line 1:

for: '...a concentration of 0·001...'

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Krassilnikoviae: A New Class of Micro-Organisms found in Sea and Ocean Depths

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SUMMARY: A new class of micro-organisms, the Krassilnikoviae, found in the Black Sea, the Pacific and the Arctic Oceans, is described. These organisms developed on slides immersed in the waters; they have not been cultivated in the laboratory. They consist of non-branching, non-septate filaments with a head at one end consisting of a cluster of round bodies. The filaments often become interlaced and form strings which can just be seen by the naked eye. They may or may not be contained in a sheath.

During an expedition to the Pacific Ocean on the S.S. *Vitijaz* a number of slides were immersed for 8–25 hr. at various depths from 50 to 1000 m. near the Kurile Islands. Upon removal a peculiar filamentous micro-organism with heads composed of a cluster of rounded bodies was found on the surface of these slides. Subsequent deep-water investigations in the Black Sea, the Pacific Ocean and the Arctic Ocean (near the North Pole) showed that this form of micro-organism was widely spread. They developed on the slides immersed in the oxygen zone and at maximum depths in the Black Sea, where the hydrogen sulphide concentration was very high. These organisms could not be cultivated from many hundreds of water samples taken at the same stations and the same horizons where they had been found on slides, and only in single cases could they be found on membrane filters through which 15–50 ml. of the same water had been passed. In sea water therefore these micro-organisms, as a rule, do not occur in a free-floating state, but only develop their characteristic forms when attached to objects suspended in the water.

METHODS

Glass slides, cleaned with ethanol or chromic-sulphuric acid mixture, washed with distilled water, dried and flamed, were inserted at one end into slits in large rubber bungs 5–6 cm. diameter. The bungs were then fixed to a cable and immersed at various horizons for periods ranging from a few hours to several days. The slides were then removed, dried, fixed by heating or in absolute ethanol, and stained with 1 % (w/v) erythrosine in 5 % (w/v) phenol aqueous solution, or with 5 % (w/v) erythrosine in 3 % (w/v) phenol aqueous solution. The morphological examination of the organisms was carried out at a magnification of × 1350. Counts of the entire slide, except the portion in contact with the bung, were also made at a magnification of × 300, a filament with a cluster head being taken as a single organism. In large and dense accumulations only separate filaments with heads were counted. Forms with a large well-formed sheath were counted separately from organisms with thin poorly staining filaments. The total number of the slides studied was over 200.

Attempts were made to grow these organisms from water samples as follows: glass slides (35 mm. × 8 mm. and 75 mm. × 12 mm.), cleaned as above, were placed in 25 and 250 ml. glass-stoppered flasks. After autoclaving, the flasks were completely filled with water from the bathometers immediately these had been removed from different depths in the Pacific Ocean or the Central Polar basin. The flasks filled with water, with the slides immersed inside, were stored at 0–2° or at room temperature for 2, 4 and 6 months, $1\frac{1}{2}$ and $3\frac{1}{2}$ years. The slides were then removed, fixed, stained with erythrosine, and examined. Altogether 142 such slides were examined.

RESULTS

The morphology of filamentous-cluster organisms

Up to the present all attempts to cultivate these organisms in the ordinary nutrient media of complex, organic or mineral composition have failed. Their morphology therefore had to be studied directly on fixed and stained slides, and by the phase-contrast and anoptral microscopes. More than 200,000 individuals (filaments with heads) from the Black Sea, the Pacific Ocean and the North Pole region have been examined. Structurally these micro-organisms are non-branching, non-septate homogeneous filaments with a head at one end, consisting of a bunch-shaped aggregate or cluster of rounded bodies which strongly refract light.

Filaments. Filaments with cluster heads were observed either singly or more often as large aggregates (Pl. 2, fig. 1). These aggregates were distributed either close to the edges or spread across the slide in thin bands forming a coating resembling frosting, visible to the naked eye. In many cases the aggregates were uniform in make-up, consisting of filaments of the same size and form with similar round bodies, but groups of mixed large and small forms also occurred. The filaments in aggregates sometimes become interlaced or even formed 'plaits' (Pl. 1, fig. *d*; Pl. 2, fig. 2). In a few cases the adhesion of several filaments was observed, but in no case was genuine branching seen.

Accumulations of filaments with cluster heads were found around knobbly strings up to 8 μ thick (Pl. 2, fig. 3). On such a string several thousand filaments with heads were sometimes found, attached by their free ends. Separate filaments were often seen lying alongside.

The filaments carrying heads were either very thin (0·4–0·5 μ) and stained poorly with erythrosine (Pl. 1, figs. *c*, *d*, *e*, *m*; Pl. 3, figs. 10, 14) or they were thick (1·2–2·5 μ), distinctly contoured, homogeneous, and stained well with erythrosine. Presumably the thick filaments consisted of thin filaments enclosed in a sheath of organic composition. This supposition is based on the fact that very often the thin and paler-staining filaments emerged from a thick one, as if from a sheath. The protruding thin thread sometimes reached a considerable length (Pl. 1, figs. *k*, *n*, *o*, *w*, *y*, *z*; Pl. 2, fig. 5) or was very short

(Pl. 1, figs. *h*, *p*; Pl. 2, fig. 4). The free end was sometimes split in two (Pl. 1, figs. *g*, *h*, *y*).

The thick and the thin filaments usually had one thickness throughout their entire length, but sometimes the sheath gradually thickened in the head direction (Pl. 1, figs. *g*, *k*, *o*, *w*; Pl. 2, fig. 5; Pl. 3, fig. 7) or formed funnel-shaped broadenings similar to apophysis in mucors (Pl. 1, figs. *f*, *i*; Pl. 4, fig. 20). Plate 1, fig. *p* and Pl. 4, fig. 22, represent forms whose sheaths were fairly thin (0·6–0·7μ).

Filaments were sometimes many microns in length, in some cases they reached tenths of a millimetre, but no correlation was observed between length and thickness. They were often twisted into spirals or rings (Pl. 1, figs. *q*, *s*, *u*; Pl. 2, fig. 6; Pl. 3, fig. 8; Pl. 4, fig. 18).

Cluster heads. The cluster head consisted of a great number (up to several score) of rounded or oval-shaped bodies, the size varying in different organisms from 0·5 to 2·0μ. Their shape was not quite regular, and in some cases a sort of dent was seen on their surfaces. There were no major variations in the dimensions of these bodies, but two types were observed in one bunch. The first included large (1·5–2·0μ) bodies of somewhat oval form, staining brightly with erythrosine; the second, smaller ones (0·8–1·0μ), stained poorly and were of greenish tint and distinctly contoured. In a photograph the latter look like rings or flat plates with a dent in the middle (Pl. 2, fig. 5; Pl. 3, fig. 7). In some cases the capsules of the bodies were rather dense so that under the microscope they gave the appearance of being hollow. This was still more evident with special staining methods.

The bodies were arranged in the heads in different ways. In some cases they were very dense and the head appeared spherical (Pl. 1, figs. *g*, *p*, *w*; Pl. 2, fig. 6; Pl. 3, fig. 11), in others less dense (Pl. 3, figs. 12, 13; Pl. 4, fig. 18). Bunches of bodies spread along the longitudinal axis were rather rare (Pl. 1, fig. *x*; Pl. 3, fig. 14). Frequently they were arranged along the periphery of the head as if connected to a broader end of the filament by means of thin strings with vague outlines, the entire formation resembling an open umbrella or a fan (Pl. 1, figs, *e*, *f*, *i*, *m*; Pl. 4, figs. 15–17, 20). Sometimes these formations had only a small number of bodies or none at all, and only a thin net, usually badly stained, was seen (Pl. 1, figs. *t*, *v*; Pl. 2, fig. 4; Pl. 4, fig. 19). Such net structures were sometimes free without any connexion with filament (Pl. 3, fig. 9). These structures may represent slime, in which the main mass of bodies lay. Thin threads, sometimes branched, at the extremes of which rounded bodies were arranged (Pl. 1, figs. *l*, *r*; Pl. 4, fig. 21) were not infrequently seen, extending from the head-end of the main filament.

All the heads appeared to be composed according to a common plan, the round bodies on the periphery of a central part filled with thin filaments or strings which formed a sort of net. The adhesion of the bodies was not strong, and they were sometimes seen outside the heads on an ordinary branch (Pl. 1, fig. *a*) or separately scattered singly upon the slide. From some of them thin filaments extended, usually longer than the head strings (Pl. 1, fig. *b*).

Two development cycles for these organisms may be suggested from the patterns seen on the slides. (1) A round body, coming into contact with a solid substrate, produces a thread which attaches the body to the substrate. Propagation is by division or by gemmation until the bodies form the characteristic cluster head on a stem. (2) The round bodies, after separating from the head, on maturation grow into thin gradually elongating threads. Then bulges appear at one end from which reproductive bodies (conidia) fragment, the latter maintaining for some time their ties with the filament branches. In this case the head represents a sort of fruit-body consisting of conidia.

Sheaths. The role of the sheath is still obscure. There was a pronounced divergence between the filaments and the sheaths in their capacity to stain with erythrosine; the filaments stained badly and the sheaths very well. The sheaths and the round bodies of the head also differed when stained by Gram's method, by fuchsin, or by Scheffer's method. Sheaths and round bodies stained Gram-positive, but only the outer layers of the round bodies stained; the unstained central part gave the hollow appearance mentioned previously. Stained with hot fuchsin (the usual spore stain), the filaments stained badly whereas the round bodies stained well.

The Scheffer stain, which stains bacteria yellowish red with safranine and spores dark-green with malachite green, stained the filament yellowish red, though less brightly than with bacteria, and the round bodies were dark green. It should be noted for comparison that Actinomyces spores are not stained with malachite green.

Formation and development

The rate of formation of the filamentous-cluster organisms was surprisingly high. Filaments with heads were found in great numbers on the slides after 8 hr. immersion at various depths in the Pacific Ocean, and a considerable number of these organisms were observed on slides exposed only for 6 hr. at various depths in the Central Polar Basin during the investigations conducted on the drifting Station 'Severnji Polus-4'. The absence of these organisms from the membrane filters through which water samples taken from the same horizons had been passed, showed that the organisms developed on the slides, and did not attach themselves from the water as ready-formed filaments with heads. Ultrafiltration of water samples produced the so-called 'cocci with thickened membrane' (Kriss & Rukina, 1952*a*) as well as conventional bacterial cells.

A comparison showed that round bodies from the heads were similar to the said cocci in their form and size, but that the latter possessed a thickened membrane which the former usually lacked. However, among the scattered heads, round bodies with a more compact membrane did occur. There was evidence that the thickness of the membrane increased as the round bodies matured.

It is interesting to note that the development of filaments with heads did not occur on any of the slides immersed in experimental flasks filled with water obtained from any depth in the Pacific and Arctic Oceans.

Distribution

The experiments with submerged slides were carried out in the Black Sea in 1954, in the Caspian Sea in 1952, in the Pacific Ocean in 1951 and 1953 and in the Central part of the Arctic basin in 1954, 1955 and 1956. The slides were suspended at standard horizons from the surface to the bottom layers. In Table 1 are summarized the data on the quantitative studies of these microorganisms at the depths investigated. The table shows that in the Black Sea the habitat was not confined to the oxygen zone. The organisms developed on slides immersed in the depths of the hydrogen sulphide zone, and large numbers were found at 500, 750, 1000, 1250 and 2000 m. The greatest number was found in the 25–150 m. layer, while the 10 m. horizon yielded only two thin filaments with heads. All the deeper levels produced organisms with sheaths.

In the Pacific Ocean the slides were put down at five stations near the north-east coast of Asia. Except for one surface and two deep slides the rest were coated with organisms, the numbers running into the thousands, varying with stations and horizon to a depth of 5500 m. (Table 1).

One difference between the Black Sea and the Pacific Ocean was that organisms without sheaths were found at all depths in the latter, not infrequently their number being considerably larger than that of sheath formers. The Pacific Ocean forms also had a greater morphological diversity.

In the region of the North Pole (Severnji Polus-3) these organisms were only found at certain horizons and not everywhere in such quantities as in the Pacific Ocean. The higher content in the upper (200 m.) and lower (1000 m.) boundaries of the Atlantic current is not accidental, as other microbial forms occur in these horizons in greater numbers than in the higher or lower levels (Kriss, 1955). It is noteworthy that the slide put down to 3300 m. counted over 16000 organisms, all with large sheaths (length up to 250μ and thickness 1·0–1·2μ), while in some horizons only thin filaments without sheaths were observed. Farther south in latitude 83° N. (Severnji Polus-5) the organisms were found in numbers which approached those of the Pacific Ocean, for both sheath-free and sheath-forming forms (see Table 1).

As the different numbers recorded at Severnji Polus-3 and Severnji Polus-5 could hardly be explained by the different times of exposure (24 and 60 hr.) a series of experiments were carried out at the station Severnji Polus-4 in which slides were exposed for 6, 12, 24 hr., or 7 days. These showed that the lengthening of the exposure to 7 days by no means contributed to the accretion of the organisms on the slides, nor to a decrease in the empty horizons. Moreover, a number of 6 hr. slides contained more organisms than the 7-day ones, and empty horizons were fewer in the 6 hr. exposures.

The above facts can be explained as follows. The drift during the investigation on the drifting station Severnji Polus-4 was negligible, and the slides hardly moved in the water. The investigations on Severnji Polus-3 were carried out under similar conditions and the station hardly moved during the 24 hr. The experiments on the drifting station Severnji Polus-5, on the other hand,

Table 1. *Distribution of filamentous-cluster organisms in the sea*

The figures are the total number of filaments with cluster heads on a glass area 15 cm.² exposed in the water for the time and depth stated. The numbers in parentheses are the numbers of forms with sheaths.

Stations:	The Black Sea (1954)	The Caspian Sea (1952)				Central Polar Basin						The Pacific Ocean					Lake Baikal (1957)
		St. 1			St. 83	SP-3 (1954)	SP-5 (1955)	SP-4 (1956)				1951			1953		
												St. 834	St. 835	St. 836	St. 10	St. 24	
Co-ordinates:	90 miles to south-west from Novorossiisk	41° 45′ N., 50° 13′ 5″ E.			40° 18′ 5″ N., 50° 57′ 5″ E.	88° 4′ 3″ N., 151° 16′ W.	82° 55′ N., 151° 32′ E.	87° 52′ 5″ N., 188° 4′ E.				46° 36′ 3″ N., 151° 36′ 5″ E.	46° 28′ 2″ N., 151° 8′ 8″ E.	46° 30′ 8″ N., 151° 25′ 2″ E.	45° 18′ N., 156° 10′ E.	49° 21′ N., 158° 30′ E.	51° 46′ 45″ N. 105° 13′ E.
Exposure:	24 hr.	12 hr	18 hr	24 hr.	14 hr.	24 hr.	60 hr.	6 hr.	12 hr.	24 hr.	7 days	8 hr.	25 hr.	25 hr.	24 hr.	12 hr.	22·5 hr.
Depth (m.)																	
0	.	.	0	.	.	0	0	5 m. (14)	5 m.–0	5 m.–0	5 m.–0	.	.	.	0	.	0
10	2	0	0	.	0	0	33 (30)	0	0	0	0	.	.	.	.	.	0
25	(10,000)	0	0	0	20 m.–0 30 m.–0	1	(2,190)	(12,600)	0	0	(8,640)	.	.	.	71	186 (3)	0
50	(24,500)	0	0	.	40 m.–0 50 m.–0	0	10 (7)	0	6	(7,900)	0	.	.	20 (3)	6,800 (1,930)	2,410 (1,015)	0
75	(24,800)	0	0	.	60 m.–0	4	3	0	0	1	0	4	.	41		.	0
100	(28,700)	.	0	0	.	5	300	30	110	0	0	917 (25)	.	1,162	4	29,505 (835)	0
150	(23,800)	.	0	0	.	50	690 (1)	240	0	0	0	.	.	.	2,885 (105)	.	0
200	(2,930)	0	0	.	.	1,670	30 (2)	0	0	150	(50)	.	.	51	237	4870	0
250	(940)	.	0	.	.	5	3,550	0	0	0	0	.	587 (16)	.	(600)	.	0
300	(50)	0	0	.	.	0	126	70	0	0	(3,800)	.	.	119	7,000 (6,540)	2,794 (10)	0
400	.	.	0	.	.	0	60	0	25	0	0	.	.	.	203 (89)	.	0
500	(6,700)	.	0	0	.	0	270	0	0	0	0	.	46	38	79 (45)	4,285 (408)	0
600	.	.	0	0	.	23	50	.	.	.	.	.	.	.	2,408 (1,449)	3,165 (1,590)	0
750	(1,900)	.	700 m.–0	0	.	0	9,950 (9,850)	0	50	0	0	.	16 (14)	.	.	4,990 (3,420)	0
800	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	7,320 (3,297)	.
900	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4,194 (175)	.
1,000	(3,900)	.	.	.	.	(400)	0	100	17 (13)	8	0	(631)	.	.		1,418 (921)	0
1,250	(7,670)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
1,500	(310)	.	.	.	.	(10)	44 (32)	3	0	0	0	.	.	.	.	445 (276)	1350 m.–0
1,750	(650)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2,000	(2,000)	.	.	.	.	(12)	440	190	0	0	0	.	.		.	3,274 (3,148)	.
2,500	.	.	.	.	.	(70)	.	0	0	(60)	10	.	.	.	.	1,252 (1,247)	.
3,000	.	.	.	.	.	40	.	55 (47)	0	70	0	.	.	.	.	360	.
3,500	.	.	.	.	.	3,300 m.– (16,300)	.	0	0	0	(70)	.	.	.	.	8	.
4,000	.	.	.	.	.	.	.	10	0	30	.	.	.	.	.	0	.
4,500	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.
5,000	.	.	.	.	.	.	.	.	.	.	.	.	.	.		(500)	.
5,500	.	.	.	.	.	.	.	.	.	.	.	.	.	.		10	.

m. = meters.

were carried out when the ice was moving faster, and in the 2½ days it had moved a considerable distance. This would account for the fact that the results obtained at the station Severnji Polus-5 approached more closely those obtained in the Pacific Ocean than the Arctic Ocean. The investigations in the Pacific Ocean and in the Black Sea were on ships which were not anchored but allowed to drift; the submerged slides therefore made rather long journeys.

A certain role seemed to be played in the development of these organisms by the attachment to a solid substratum. Frequently macroscopically-visible strings were found at the edges of the slide, and any jags, cracks or unevenness or particles deposited upon the slides contributed to the appearance of the round bodies. This may account for the wide variations sometimes obtained; for example, on two slides suspended side by side from one cork. These organisms were not found in the Caspian Sea or in Lake Baikal at any horizon or exposure. Presumably this may be due to the inadequate salt concentration.

Cocci with a thickened membrane. The identity of these forms found in the Sea of Okhotsk, the Pacific Ocean and the Central Polar Basin (Kriss & Rukina, 1952*a*; Kriss & Birjusova, 1955; Kriss, 1955) with the round and oval-shaped bodies which compose the head of the filamentous-cluster organisms, seems to be beyond doubt. It has now become evident that a free-floating state of existence is characteristic only of the round bodies or of the 'cocci with a thickened membrane'. The membrane filters showed that water samples from various depths contained a considerable number of these cocci and almost no organisms of the type encountered on the slides. Counts from the membrane filters showed that the number of these cocci was fairly large in the ocean depths (Table 2). At some stations in the Pacific Ocean the surface layers were free from these forms or the numbers were less than in the deeper horizons. As will be seen in Table 2 they were found in the deepest levels, down to 9000 m. at Statior no. 26. In the region of the North Pole these coccus-like forms were als distributed from surface to bottom of the water body. The vertical distribution was more uniform; the surface layers contained about the same numbers as the deep horizons.

The cocci which possessed thickened membranes (cell walls) were found not only in the water of the seas but in deep bottom sediments as well. Columns of mud 1·5 to 32 m. long were obtained (Kriss & Rukina, 1952*b*) from the mud of the Okhotsk Sea, and it was found that their content of cocci with thickened membranes was higher (Table 3) deep in the mud than on the surface of the mud where sedimentation is occurring at the present time. Assuming that the formation of 1 mm. of sediment on the bottom of the Pacific Ocean requires approximately 1400 years (Bruevich, 1949), the horizons with the higher content of this type of cocci formed the surface of the bottom hundreds of thousands and even millions of years ago. Apparently, the occurrence of the filamentous-cluster organisms in the past was higher than at the present day and probably they represent ancient forms. Perhaps the conditions for their life activity are not now so favourable as they were in bygone epochs.

Table 2. *Distribution of round bodies (cocci with a thickened membrane) in the sea*

The figures shown are the numbers of cocci in 1 ml. of sea water at various depths.

Stations:	The Pacific Ocean								Region of the North Pole	
Co-ordinates:	St. 1 42° 18′ N., 150° 25′ E.	St. 3 44° 16′ N., 150° 52′ E.	St. 4 42° 59′ N., 152° 10′ E.	St. 9 44° 32′ N., 154° 51′ E.	St. 14 46° 35′ N., 157° 30′ E.	St. 17 48° 20′ N., 159° 32′ E.	St. 22 52° 24′ N., 163° 52′ E.	St. 26 44° 10′ N., 150° 30′ E.	July 88° 4′ 3″ N., 151° 16′ W.	September 89° 29′ 5″ N., 65° 43′ W.
Depth (m.)										
0	0	0	59	13	52	0	0	26	1205	1,688
10	0	0	0	38	78	0	7	0	1,096	757
25	0	0	137	33	98	0	351	7	.	392
50	72	0	104	20	98	247	65	7	1,331	548
75	176	312	176	60	65	228	65	26	1,540	209
100	111	202	468	33	52	410	72	33	1,966	426
150	241	195	273	351	351	1,086	110	117	1,514	1,070
200	410	1,398	215	449	943	462	46	78	609	966
250	280	878	163	501	1,059	234	169	624	1,253	670
300	267	904	423	650	1,053	234	136	280	1,209	557
400	.	624	371	644	683	202	410	1,755	357	653
500	546	734	403	917	637	410	273	1,378	487	522
600	338	1,638	527	975	442	429	364	1,774	1,711	.
750	624	1,410	546	1,500	247	318	2,606	1,118	1,554	748
1,000	227	1,092	1,229	1,079	423	1,268	494	1,072	1,096	357
1,500	507	2,574	2,756	1,430	364	865	351	1,579	1,392	313
2,000	201	2,353	2,815	1,417	650	904	305	1,255	853	861
2,500	293	1,540	3,270	2,870	702	1,956	2,945	1,813	1,592	444
3,000	696	579	3,029	1,606	546	813	1,176	1,736	2,419	148
3,400	.	.	.	.	.	.	.	.	374	.
3,500	.	.	.	.	.	.	.	.	.	339
3,700	.	.	.	.	.	.	.	.	496	.
4,000	780	1,534	.	9,341	455	1,755	1,852	6,305	.	.
5,000	.	2,821	.	.	.	2,009	.	6,201	.	.
6,000	.	1,372	.	.	.	.	.	5,408	.	.
7,000	.	1,950	.	.	.	.	.	11,787	.	.
8,000	.	.	.	.	.	.	.	1,398	.	.
8,500	.	.	.	.	.	.	.	2,438	.	.
9,000	.	.	.	.	.	.	.	2,366	.	.

Table 3. *Vertical distribution of round bodies (cocci with a thickened membrane) in deep bottom sediments from the sea of Okhotsk*

St. 833 44° 58′ 7″ N., 145° 24′ 7″ E. Depth (cm.)	Millions	St. 848 47° 47′ 5″ N., 151° 52′ 9″ E. Depth (cm.)	Millions	St. 875 50° 10′ 5″ N., 152° 12′ E. Depth (cm.)	Millions	St. 888 47° 33′ 2″ N., 146° 54′ 5″ E. Depth (cm.)	Millions	St. 890 46° 32′ 3″ N., 149° 10′ E. Depth (cm.)	Millions	St. 915 53° 10′ 5″ N., 148° 44′ 6″ E. Depth (cm.)	Millions	St. 917 53° 3′ 5″ N., 146° 10′ 6″ E. Depth (cm.)	Millions
.	.	0–2	36	0–1	110	0	637	0–2	151	0–2	117	0–2	104
								2–4	117				
0–4	333	2–4	136	.	.	.	.	.	.	2–4	148	.	.
												10–12	445
30–39	323	35	131	35	170	35	653	40–50	733	.	.	45	1,161
100–104	320	105	184	90	291	100	1,137	.	.	.	.	105	2,421
166	357	155	182	170	1,268	175	1,255	.	.	.	.	175	1,957
217–225	296	.	.	230	1,329	255	1,465	.	.	.	.	255	2,449
300–305	495	.	.	.	.	.	.	.	.	.	.	315	2,130
								350–355	236	.	.	385	1,362
								.	.	.	.	445	1,773
								680–688	488	702–704	274	495	1,255
								.	.	1,035–1,038	738	.	.
								1,145–1,150	401	.	.	.	.
								.	.	1,222–1,225	767	.	.
								1,975–1,980	386	.	.	.	.
								2,340–2,350	432	.	.	.	.
								2,725–2,730	675	.	.	.	.
								3,195–3,200	612	.	.	.	.

Systematic position

The structure of the filamentous-cluster organisms is peculiar and they do not fit into any of the known classes of lower organisms. Excluding protozoa, algae and fungi, the rest of the lower forms fall into four classes (according to the system of Krassilnikov, 1949): Actinomycetes, Spirochaetae, Eubacteriae and Myxobacteriae. In contradistinction to Actinomycetes, typical representatives of which are characterized by the formation of a mycelium, the filamentous-cluster organisms lack the ability to form branching filaments. Neither short nor long filaments carrying the bunch-shaped head have side branches.

Table 4. *The major differences between the new class and the classes according to the system of Krassilnikov* (1949)

Krassilnikoviae	Actinomycetes	Eubacteriae	Myxobacteriae	Spirochaetae
Filaments, non-septate, non-branched (non-ramified), enclosed in a sheath or lack it. At one end form a bunch-shaped head, consisting of rounded bodies	Cells mycelial, or rod- or coccus-shaped. Mycelium septate or non-septate, branched (ramified). Form spores arranged singly or in chains in the air-hyphae	Cells rod-, coccus-, spiral- or filament-shaped, spore-forming or none. True ramification absent. Filamentous organisms have numerous septa	Cells rod- and coccus-shaped, motile, changing form while moving. Create 'fruit bodies' and cysts	Cells of spiral form, motile

The absence of mycelium and the much smaller filament diameter differentiates these organisms from the fungi, and further they have no basal features in common with the fungi which have reduced forms of mycelium, e.g. such a form as *Aphanomycopsis bacillariacearum* (Scherffel, 1925) of the family Saprolegniaceae.

The presence of filaments in the filamentous-cluster organism distinguishes it from the spirochetes, and though they resemble some members of the order Chlamydobacteriales, the filamentous multicellular organisms of the class Eubacteriae (Krassilnikov, 1949), this resemblance is only superficial. Two important characters distinguish the two groups clearly, namely the formation of a head at one extreme of the filament and the non-septate filament of the filamentous-cluster organisms; neither of these characters is seen in the Chlamydobacteriales.

The ability to form a bunch-like head attached to a string and surrounded by a sheath is a conspicuous feature of the described organisms; this places them apart from organisms like *Gallionella* and *Siderocapsa* or *Perseus marinus* described by Butkevich (1928). The filamentous-cluster organisms cannot be considered as belonging to the class Myxobacteriae either, since they do not form cysts or 'fruit bodies'.

The outward appearance of the filamentous-cluster organisms is similar to that of *Anthophysa vegetans* from the family Monadaceae (Eyferth, 1925).

This organism forms heads attached to a long stem; the head consisting of scores of pear-shaped cells, possessing 'tails'; the diameter of the cell-monads is about 30μ and they have an obvious inner structure and the stem is usually ramified fairly well. The differences between these two types of micro-organisms are conspicuous.

Undoubtedly as far as their structure is concerned, the filamentous-cluster organisms form a new class of micro-organisms occupying an intermediate position between the protozoa and the bacteria. We name this new class of micro-organisms after Professor N. A. Krassilnikov.

> *Diagnosis*: filaments, non-septate, non-ramified, diameter $0{\cdot}4$–$0{\cdot}5\mu$, may be enclosed in a sheath. At one end form a cluster-shaped head, consisting of rounded bodies of diameter $0{\cdot}5$–$2{\cdot}0\mu$. The number of round bodies in a cluster on one filament may amount to several scores. The organisms are widely spread in seas and oceans. May be found in considerable number at deep horizons. Have not been obtained in laboratory culture. Do not grow in conventional media or in isolated sea water samples. Rapidly develop on submerged fouling slides.

REFERENCES

Bruevich, S. (1949). Rate of formation of bottom sediments in world's ocean. *Trud. Inst. oceanolog.* **3**, 90 (in Russian).

Butkevich, V. (1928). Formation of marine ferrous-manganese sedimentations and microorganisms participating in it. *Trans. oceanogr. Inst. Moscow*, **3**, 7 (in Russian).

Eyferth, B. (1925). *Einfachste Lebensformen des Tier-und Pflanzenreiches*, **1**, 249, Berlin: Bermühler.

Krassilnikov, N. (1949). Determination of *Actinomycetes* and bacteria. *Isdatelstvo Acad. Sci. U.S.S.R.* Moscow-Leningrad, p. 830 (in Russian).

Kriss, A. (1955). Microbiological investigations in the region of the North Pole. *Vestn. acad. Sci. U.S.S.R.* **1**, 30 (in Russian).

Kriss, A. & Birjusova, V. (1955). Vertical distribution of organisms in the Kurile-Kamchatka trench of the Pacific Ocean. *C.R. Acad. Sci. U.S.S.R.* **100**, 1175 (in Russian).

Kriss, A. & Rukina, E. (1952*a*). Biomass of microorganisms and its rate of multiplication in ocean depths. *J. gen. Biol., Moscow*, **13**, 346 (in Russian).

Kriss, A. & Rukina, E. (1952*b*). Microorganisms in bottom sediments of ocean regions. *Bull. Acad. Sci., U.S.S.R.* (*biology*), **6**, 67 (in Russian).

Scherffel, A. (1925). Endophytische Phycomyceten-Parasiten der Bacillariaceen und einige neue Monadinen. *Arch. Protistenk*, **52**, 1.

EXPLANATION OF PLATES

Plate 1

Microbial forms observed in the Pacific Ocean: *a, b, f, g, h, i* = at the depth 700 m.; *c* = at 400 m.; *k, n, o,* = at 2500 m.; *l* = at 900 m.; *q, t* = at 25 m.; *r* = at 5000 m.; *v* = at 1000 m.; *w* = at 50 m.; *x* = at 200 m.; *y* = at 3000 m.; *z* = at 800 m. Microbial forms observed in the ocean in the region of the North Pole: *d* = at 1000 m.; *e* = at 300 m.; *m* = at 3000 m.; *p* = at 200 m.; *s, u* = at 25 m.

PLATE 2

Fig. 1. From 25 m. depth in North Pole region. Filamentous-cluster organisms. ×450.
Fig. 2. From 25 m. in North Pole region. Interlaced filaments. ×1600.
Fig. 3. From 5000 m. in Pacific Ocean. Accumulation of filaments with cluster heads around knobbly string. ×450.
Fig. 4. From 5000 m. Pacific Ocean. Short thread protruding from sheath. A thin net without round bodies. ×1600.
Fig. 5. From 2500 m. in Pacific Ocean. Long thread protruding from sheath. The latter gradually thickened in the head direction. Two types of round bodies. ×1600.
Fig. 6. From 900 m. in Pacific Ocean. Filament twisted into spirals. ×1600.

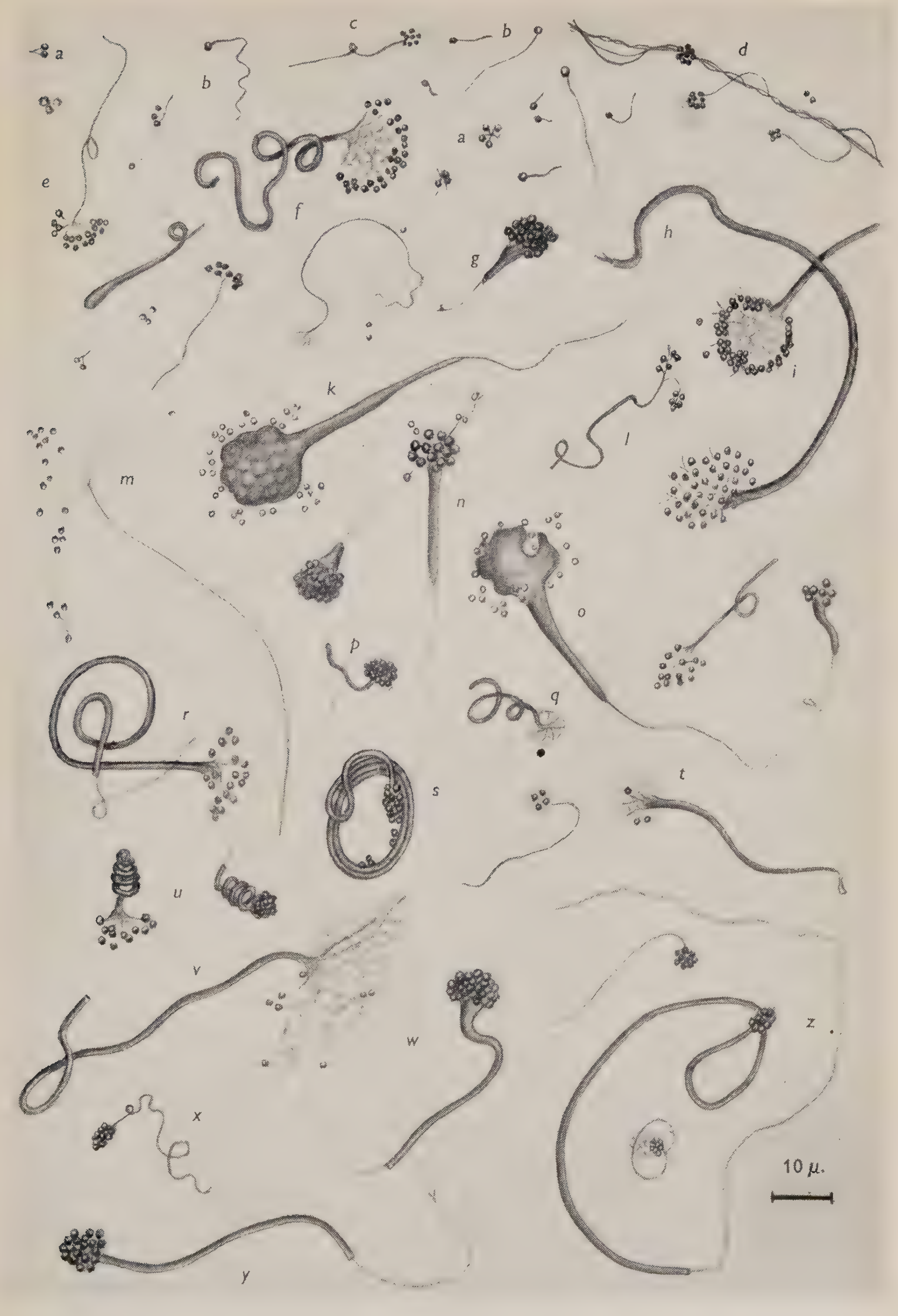
PLATE 3

Fig. 7. From 2500 m. in Pacific Ocean. Round bodies looking like flat plates with a dent in the middle. ×1420.
Fig. 8. From 25 m. in North Pole region. Filament twisted into rings. ×1420.
Fig. 9. From 750 m. in North Pole region. Net structures without any connexion with filament. ×1420.
Fig. 10. From 3000 m. in North Pole region. Thin filament without a sheath. ×1420.
Fig. 11. From 750 m. in North Pole region. Bodies arranged very dense. ×1420.
Fig. 12. From 25 m. in North Pole region. Bodies arranged in the head not so compact as on fig. 11. ×1420.
Fig. 13. From 5000 m. in Pacific Ocean. Bodies arranged not compactly. ×1420.
Fig. 14. From 3000 m. in North Pole region. Thin filament. Bunch of bodies spread along the longitudinal axis. ×1420.

PLATE 4

Fig. 15. From 25 m. in North Pole region. Thin strings which connected the bodies with the filament. ×1330.
Fig. 16. From 700 m. in Pacific Ocean. Bodies arranged in the head. ×1330.
Fig. 17. From 600 m. in Pacific Ocean. Bodies arranged in the head. ×1330.
Fig. 18. From 250 m. in Pacific Ocean. Bodies arranged in the head. ×1330.
Fig. 19. From 600 m. in Pacific Ocean. Thin net in the head. ×1330.
Fig. 20. From 900 m. in Pacific Ocean. Broadening of the filament in the head. ×1330.
Fig. 21. From 700 m. in Pacific Ocean. Thin threads at the extremes of which round bodies are arranged. ×1330.
Fig. 22. From 900 m. in Pacific Ocean. Filament with thin sheath. ×1330.

(*Received* 29 *April* 1958)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A. E. Kriss & I. N. Mitzkevich—The Krassilnikoviae. Plate 1

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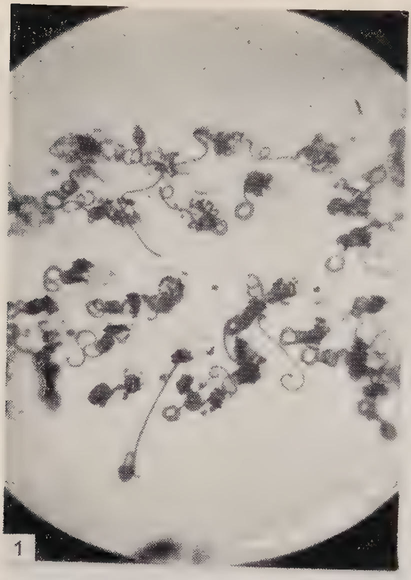
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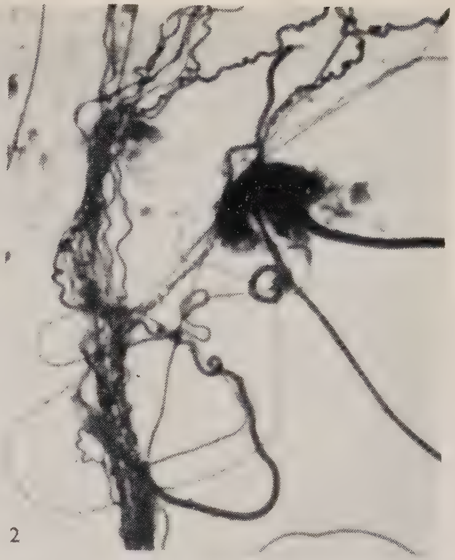
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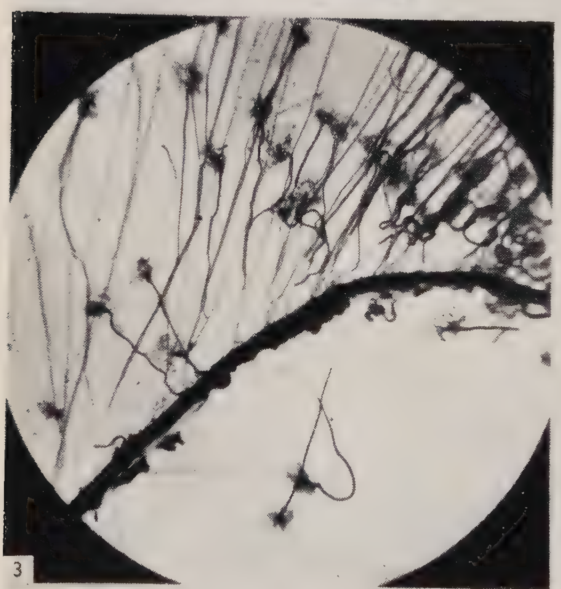
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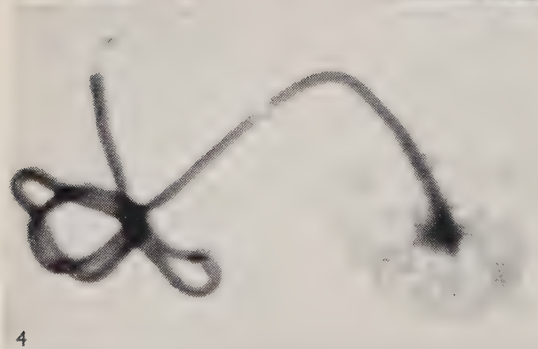
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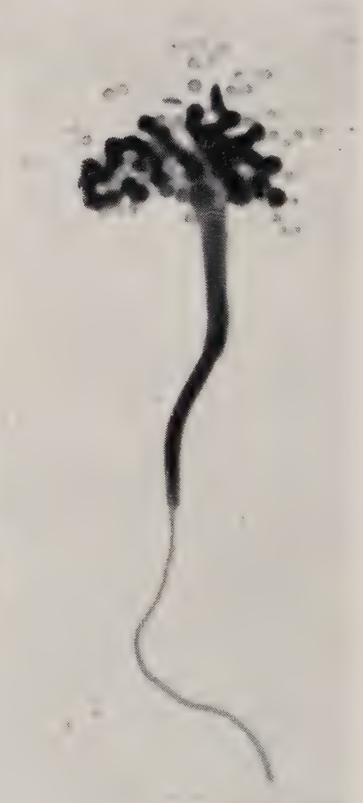
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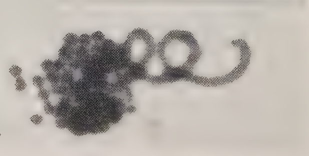
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A. E. Kriss & I. N. Mitzkevich—The Krassilnikoviae. Plate 2

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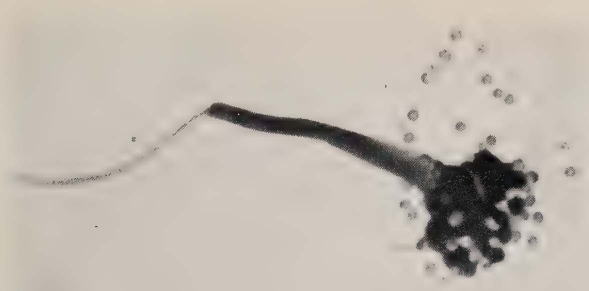

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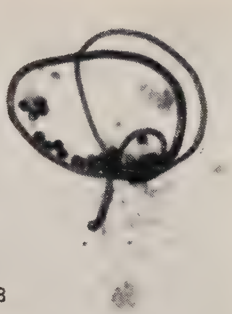

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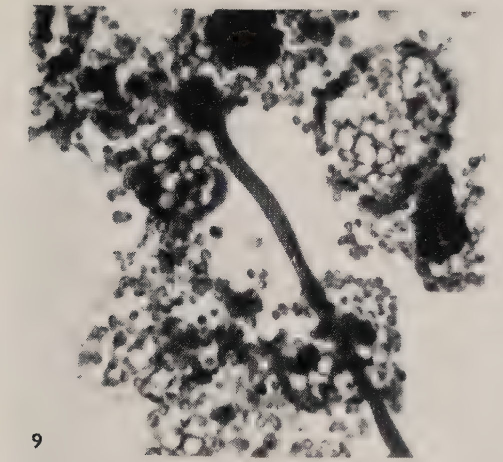

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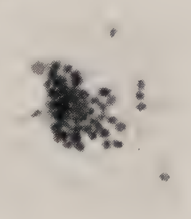

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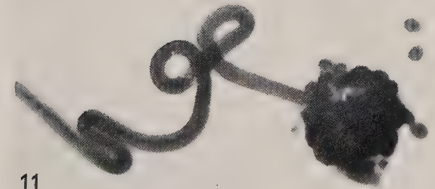

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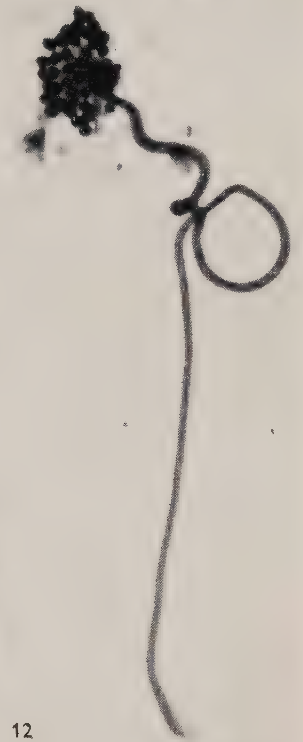

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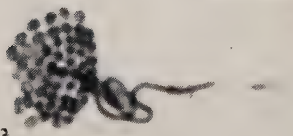

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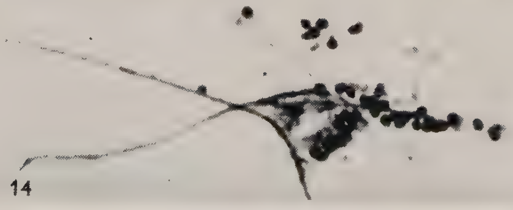

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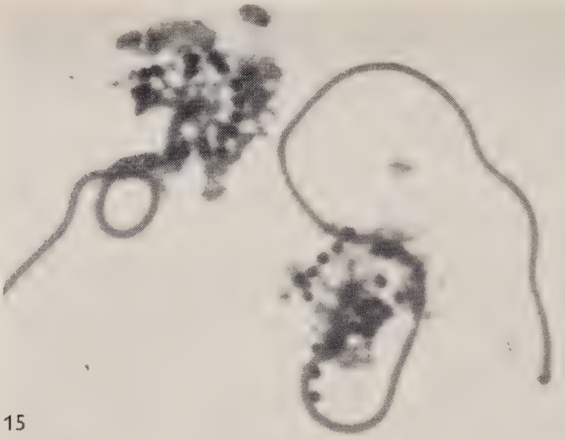
A. E. Kriss & I. N. Mitzkevich—The Krassilnikoviae. Plate 3



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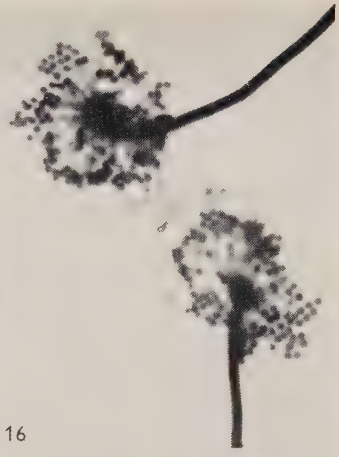

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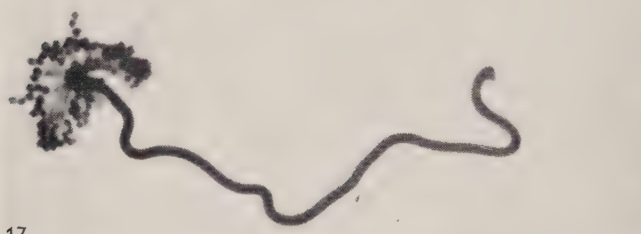

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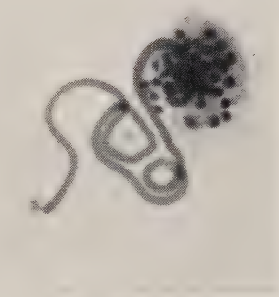

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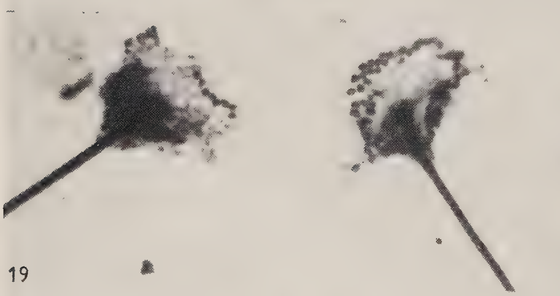

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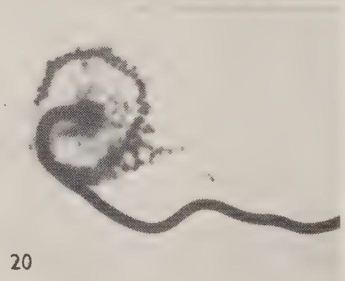

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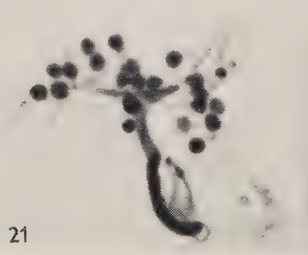

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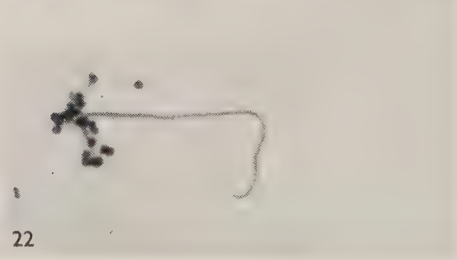

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A. E. Kriss & I. N. Mitzkevich—The Krassilnikoviae. Plate 4

MENNA, M. E. DI (1959). *J. gen. Microbiol.* **20**, 13–23

Some Physiological Characters of Yeasts from Soils and Allied Habitats

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SUMMARY: An investigation has been made of some physiological properties of yeasts from soil and from the leaf surfaces of pasture plants. Almost all the species tested could use cellobiose, and the majority could synthesize growth factors and use a range of organic acids as carbon sources. Some species, including all the pigmented ones examined, could use aromatic compounds as carbon sources. A few species could liquefy gelatin. Only two were able to degrade pectin completely under the conditions used. Almost all could grow within a wide range of hydrogen ion concentrations and at osmotic pressures of at least 30 atmospheres. Ability to survive desiccation varied greatly. Only one species showed any ability to grow at low oxygen tensions. No marked or consistent differences could be seen between soil and leaf yeasts. As both groups are competent to grow in substrates which supply low concentrations of complex foodstuffs, it seems likely that antagonistic factors originating from other members of the soil and leaf populations are responsible for keeping the two floras distinct.

There appear to be relatively few species of yeasts which occur in important numbers in New Zealand soils (di Menna, 1955*a*, 1957, 1958; A. C. S. Wright, to be published). An examination has been made of some of the physiological characters of these species in an attempt to discover what part they play in the organic cycle of soil, to find some of the limiting and predisposing factors which may influence their distribution, and to find the lines upon which more intensive research may best be followed. Some yeast species from the leaf surfaces (phyllosphere) of pasture plants (*Lolium perenne*, *Trifolium repens*, *Anthoxanthum odoratum* and *Agrostis tenuis*) are also included in the work. The species selected had been on one or several occasions the dominant yeast species in cultures from soil suspensions or leaf washings on acidified glucose peptone agar, or they had been frequently found in these habitats, although in smaller proportions. In addition, a number of species of uncertain habitat, but possibly of importance in a soil or phyllosphere flora were included in the work.

Schizoblastosporion starkeyi-henricii was dominant in three of five samples in one collection of soil (di Menna, 1957), but although samples were taken on a number of occasions from the same site it was never again found in large proportions and has been isolated only sporadically from other soils. About 20 % of the air-borne yeasts collected in Dunedin, New Zealand (di Menna, 1954, 1955*b*) were *Debaryomyces* spp., mainly *D. kloeckeri*. The habitats of all the other common air-borne yeasts of these surveys were found to be soil or leaves. As it seems likely that one or both of these substrates will prove to be the source of *Debaryomyces* spp., representatives of two members of the genus were included in this work. *Cryptococcus luteolus*, *Candida guilliermondii* and *Sporobolomyces pararoseus* had appeared in cultures from soil and grass irregularly and in small numbers but sufficiently frequently to warrant inclusion in this work.

The yeasts examined were: from soil *Saccharomyces delbrueckii* Lindner, *Cryptococcus albidus* (Saito) Skinner, *C. diffluens* (Zach) Lodder & Kreger-van Rij, *C. terreus* di Menna, *Candida tropicalis* (Cast.) Berkhout, *C. humicola* (Daszewska) Diddens & Lodder, *C. parapsilosis* (Ashf.) Langeron & Talice, *C. curvata* (Diddens & Lodder) Lodder & Kreger-van Rij, *C. muscorum* di Menna, *Trichosporon pullulans* (Lindner) Diddens & Lodder, *T. cutaneum* (de Beurm., Gougerot & Vaucher) Ota; from the phyllosphere *Sporobolomyces roseus* Kluyver & van Niel, *Cryptococcus laurentii* (Kufferath) Skinner, *Torulopsis ingeniosa* di Menna, *Rhodotorula glutinis* (Fres.) Harrison, *R. mucilaginosa* (Jorg.) Harrison, *R. graminis* di Menna. Of uncertain habitat *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij, *D. kloeckeri* Guill. & Peju, *Sporobolomyces pararoseus* Olson & Hammer, *Cryptococcus luteolus* (Saito) Skinner, *Candida guilliermondii* (Cast.) Langeron & Guerra, *Schizoblastosporion starkeyi-henricii* Ciferri.

Usually two isolates of each species were examined. Only one isolate each of *Debaryomyces hansenii*, *Sporobolomyces pararoseus*, *Cryptococcus luteolus*, *C. diffluens*, *Candida tropicalis*, *Trichosporon pullulans* and *Rhodotorula mucilaginosa* was available for some or all of the tests. Species had been identified by the methods of Lodder & Kreger-van Rij (1952). These authorities use routinely in their identifications the fermentation of glucose, sucrose, maltose, lactose, galactose and raffinose, and the assimilation of glucose, sucrose, maltose, lactose, galactose and potassium nitrate. Production of starch-like compounds was also looked for in certain groups. The term 'fermentation' as used here means the production of acid and of gas in large enough quantities to be trapped in a Durham tube. The fermenting species studied were *Saccharomyces delbrueckii*, *Candida tropicalis*, *C. guilliermondii* and *C. parapsilosis*. 'Pigmented species' referred to later in this work were the species of *Sporobolomyces* and *Rhodotorula* used.

The physiological characters studied were:

(1) Physico-chemical. The limits of hydrogen-ion concentration, osmotic pressure and oxygen tension within which growth occurred, and ability to survive desiccation.

(2) Nutritional; ability to use organic acids, aromatic compounds, cellobiose and cellulose as sole carbon sources; pectinolytic and amylolytic activity; proteolytic activity; effects of decreasing the concentration of (*a*) carbon source, (*b*) nitrogen source, (*c*) growth factors, and (*d*) mineral nutrients.

METHODS

Two-day-old cultures on glucose peptone agar were used for inoculations. All cultures were incubated at room temperature. Two media were used extensively, glucose yeast extract broth (Kurung, 1942) and the liquid auxanographic medium of Lodder & Kreger-van Rij (1952, p. 25).

Physico-chemical tests

Hydrogen-ion concentration. The pH values of three batches of glucose yeast extract broth was adjusted by the addition of N-HCl to pH 2, 3 and 4. The pH of three batches of peptone water (1 %, w/v, peptone, British Drug Houses Ltd., in tap water) was adjusted with NaOH to values of 8, 9 and 10, glucose yeast extract broth having proved unsuitable in this range. Results were read a week after inoculation for pH values of 2, 3, 4, and at 3 days after inoculation for values of 8, 9 and 10.

Osmotic pressure. Glucose yeast extract broth at pH 4 was made up without NaCl. The broth was assumed to have an osmotic pressure of *c.* 5 atmospheres (8 g. glucose/l. distilled water gives a calculated osmotic pressure of 1 atmosphere). Glucose was added to give media with calculated osmotic pressures of 10, 20, 30, 40, 60, 80 and 100 atmospheres. A second series of media was prepared in which NaCl was added to give calculated osmotic pressures of 10, 20, 30, 40, 60 and 80 atmospheres. 2·6 g. NaCl/l. distilled water was taken to give an osmotic pressure of 1 atmosphere. Cultures were incubated for a week before the results were read.

Oxygen tension. Two media were used: (*a*) 1 %, w/v, peptone in tap water solidified with agar (1 %, w/v, agar, Davis (N.Z.) Ltd., standard grade); (*b*) 15 %, w/v, gelatin, Davis (N.Z.) Ltd., in tap water. The tubed medium was melted, cooled to *c.* 45°, inoculated, and mixed by rolling without shaking to avoid inclusion of air. Cultures were incubated for 2 weeks; ability to grow at decreased oxygen tensions was measured by the depth to which growth had occurred.

Desiccation. Yeasts in sterile tap water were dropped on sterile filter-paper disks and dried out at room temperature in a dark, well-ventilated cupboard without the use of desiccants. The disks appeared dry in a week and were stored in air-tight containers. Viability was tested by incubating a disk in a tube of glucose yeast extract broth, at first at weekly intervals, but later at two-weekly, and finally at monthly intervals. A yeast was considered no longer viable when three successive cultures were sterile.

Nutritional tests

Use of carbon sources. Liquid 'auxanographic' medium in which glucose was replaced by the carbon compounds to be tested was used for this series of experiments. The organic acids used (see Table 2) were added in 0·1 % (w/v) concentration, the medium brought to pH 4–5 with NaOH and sterilized by autoclaving at 15 lb./sq.in. for 15 min. The volatile aromatic compounds, ferulic acid and *p*-hydroxybenzaldehyde (L. Light and Co.) and vanillin (British Drug Houses Ltd., A.R.), which are products resulting from the degradation of lignin, were added to the sterile basal medium in 0·01 % (w/v) concentration and left for some weeks to test sterility before dispensing and inoculating. Cellulose and cellobiose were added to 0·5 % concentration, the medium solidified with 2 % agar and sterilized by autoclaving. Solidification of this series was necessary because of the particulate nature of cellulose powder. Control tubes of basal medium without a carbon source were inoculated in each series of cultures. All results were read after incubation for 2 weeks.

Proteolytic activity. Proteolytic activity was tested with gelatin and egg albumin. The 15 % gelatin tubes used for detection of growth at decreased oxygen tensions were incubated for 4 weeks and examined for proteolysis.

Egg albumin slopes were prepared as follows: egg flake albumin (British Drug Houses, Ltd.) 7·5 % (w/v); glucose 1 %; KH_2PO_4 0·1 %, in tap water, mixed, dispensed in tubes, and steamed in a sloping position for 20 min. on 2 successive days. Cultures on these were incubated for 3 weeks.

Amylolytic activity. Soluble starch (British Drug Houses, Ltd., A.R.) was added to 0·2 % (w/v) concentration to 1 % peptone water. Three weeks after inoculation the cultures were tested with iodine for disappearance of starch. A parallel series of cultures was made on the medium of Mager & Aschner (1947) to test the production of starch-like compounds.

Pectinolytic activity. Use was made of the property of both pectin and pectic acid solutions to form a clot when mixed with ethanol. A pectin-containing medium was made as follows: minced New Zealand grapefruit were soaked overnight in three times their weight of water, boiled next day for 2 hr., and filtered through muslin. The filtrate was brought to pH 4 with NaOH, dispensed and sterilized by steaming for 20 min. on 3 successive days. After incubation for 6 weeks an equal volume of ethanol was added to the cultures. Absence of a gel indicated pectinolysis.

Effect of decreasing the concentration of nutrients

Carbon and nitrogen sources. Two series of auxanographic media solidified with agar (2 %, w/v) were prepared. In one series six concentrations of the carbon source (glucose) were used: 0·5 % (standard), 0·2, 0·1, 0·05, 0·01 % and nil. In the second series the concentration of the nitrogen source (ammonium sulphate) was varied: 0·5 % (standard), 0·2, 0·05, 0·01, 0·0025 % and nil. Inoculations were made by placing drops of the yeast suspensions upon the agar slopes in as uniform a manner as possible. Cultures were incubated for 2 weeks and the amount of growth read visually.

Mineral nutrients. The proportions of the mineral nutrient mixture in the liquid auxanographic medium was decreased from standard to 1/10 standard, 1/100, 1/1000 and nil. Particular care was taken with this series to avoid mineral contamination. All chemicals used were 'Analar' grade. Glassware was cleaned with chromic-sulphuric acid mixture and given a final rinse with triple glass-distilled water. Test tubes 18 mm. in diameter containing 10 ml. of the medium were plugged with cotton wool wrapped in Whatman's No. 50 filter-paper to prevent possible mineral contamination via cotton fibres. The cultures were incubated for 2 weeks. The optical density was measured with a Spekker photoelectric absorptiometer, using Kodak No. 5 yellow-green filters. Cultures of *Candida humicola* and *Trichosporon cutaneum*, which had granular growth forms, were homogenized with a tissue grinder before readings were made.

Growth factors. Concentration of the growth factor mixture in the liquid auxanographic medium was decreased from the standard to 1/10 standard, 1/1000 standard and nil. The optical densities of growth were measured visually.

RESULTS

Physico-chemical tests (Table 1)

Hydrogen ion concentration. All the yeasts tested grew in broth at pH values of 4 to 8; the majority grew at values of 3 and 10, but very few were capable of growth at pH 2. *Candida tropicalis, C. humicola, C. guilliermondii* and *Rhodotorula mucilaginosa* grew at all pH values tested. There was little or no variation amongst isolates of the same species.

Table 1. *Effects of some physico-chemical factors upon yeasts*

		Greatest osmotic pressure in which growth occurs		
From soil	pH within which growth occurred	Produced by glucose (atm.)	Produced by NaCl (atm.)	Duration of viability of desiccated cultures (weeks)
Saccharomyces delbrueckii	3– 8	60	30	8–10
Cryptococcus albidus	2– 8	30	30	4– 8
C. diffluens	3–10	40	20	Over 37
C. terreus	3– 8	40	30	Over 37
Candida tropicalis	2–10	60	40	4
C. humicola	2–10	60	30	2– 3
C. curvata	2– 8	30	30	7
C. muscorum	3–10	60	40	3–18
Trichosporon pullulans	4–10	40	30	8
T. cutaneum	4–10	40	10	4
From phyllosphere				
Cryptococcus laurentii	4– 9	30	20	12
Torulopsis ingeniosa	3– 9	30	20–30	3
Rhodotorula glutinis	3– 9	60	30	8–20
R. mucilaginosa	2–10	60	40	> 37
R. graminis	3–10	60	40	32
Miscellaneous				
Debaryomyces hansenii	4– 9	80	60	8
D. kloeckeri	3–10	80	60	7–16
Sporobolomyces pararoseus	3– 9	60	40	32
Cryptococcus luteolus	3– 8	30	30	3
Candida guilliermondii	2–10	80	60	13–18
Schizoblastosporion starkeyi-henricii	3– 8	20	10	0

Osmotic pressure. There was much variation in the ability of the different species tested to grow in solutions of high osmotic pressure, but little variability within species. The fermenting and pigmented species included were able to grow at higher salt and glucose concentrations than the colourless and non-fermenting species. *Schizoblastosporion starkeyi-henricii* was particularly sensitive, failing to grow at pressures above 20 atmospheres in glucose and 10 atmospheres in salt. Almost all species were more sensitive to calculated osmotic pressures produced with salt than with glucose.

Oxygen tension. None of the species was able to grow in the depths of the gelatin tubes; *Saccharomyces delbrueckii* produced a zone of growth to a depth of 8 mm. below the surface of the medium, the others grew to a depth of 2–5 mm. In the peptone agar *S. delbrueckii* grew to a depth of 5 mm. below the surface. *Candida humicola* and *Trichosporon pullulans* produced some mycelial tufts in the depths of the peptone agar; the growth of other species was restricted to the surface or to immediately beneath the surface.

Desiccation. There was considerable variation in resistance to desiccation amongst species and amongst isolates of the same species. All isolates of *Cryptococcus diffluens*, *C. terreus* and *Rhodotorula mucilaginosa* were viable after storage for 9 months. *Sporobolomyces pararoseus* and *Rhodotorula graminis* survived for 8 months in the dry state. No viable cultures were obtained from *Schizoblastosporion starkeyi-henricii* after drying.

Nutritional tests (Tables 2 and 3)

Use of carbon sources. No yeast tested was able to use cellulose as a carbon source. All but *Saccharomyces delbrueckii* and *Schizoblastosporion starkeyi-henricii* used cellobiose. All used glutamic acid and almost all used acetic, fumaric, α-keto glutaric, gluconic, citric and succinic acids; only *Saccharomyces delbrueckii*, *Trichosporon cutaneum* and *Schizoblastosporion starkeyi-henricii* or some isolates of these species failed to grow. No yeast used maleic or oxalic acid under the conditions imposed. There was considerable variety in reaction to tartaric, lactic and oxaloacetic acids. Of the species tested, only *Trichosporon cutaneum* showed much variability in assimilation of organic acids; besides the two isolates used for all tests, five other isolates of *T. cutaneum* were grown on the acids, and of the seven, three used all but maleic and oxalic acids, and the remaining four gave erratic assimilation patterns. Van Uden & Do Carmo Sousa (1957) found a similarly variable response of *T. cutaneum* to soluble starch, mannitol, inositol and sorbitol, and concluded that the species must be heterogeneous.

Torulopsis ingeniosa, *Candida muscorum*, *Trichosporon pullulans* and all the pigmented yeasts tested (with the exception of one isolate of *Rhodotorula glutinis* which will be discussed later) used vanillin, ferulic acid and *p*-hydroxybenzaldehyde. A few other non-fermenting yeasts used one or two of these compounds.

Proteolytic activity. None of the species tested was able to liquefy egg albumin although all grew well on it. *Torulopsis ingeniosa* and *Candida muscorum* liquefied gelatin rapidly (within a week), *Trichosporon cutaneum*, *T. pullulans* and *Sporobolomyces roseus* slowly, and *Cryptococcus diffluens* and *Rhodotorula graminis* slowly and variably. No odour accompanied the liquefaction of gelatin.

Pectinolytic activity. *Torulopsis ingeniosa* and *Candida humicola* were the only species which broke down the pectin preparation completely; all species grew well on the medium. Wieringa (1956), using a test for pectin hydrolysis which did not demand complete breakdown, reported that *Cryptococcus albidus* and *Rhodotorula glutinis* were pectinolytic. Bell & Etchells (1956) stated that twenty of sixty-one yeasts species and varieties were capable of de-esterifying citrus pectin. Pectinolysis is of obvious importance in the breakdown of plant materials in the soil, pectinolytic organisms probably being primary invaders (Wieringa, 1955). Further work is needed to demonstrate the presence or absence of pectinases in soil and leaf yeasts.

Table 2. *Organic acids used by yeasts*

None of the species tested used maleic or oxalic acids; all used glutamic.

	Acetic	Citric	Fumaric	Gluconic	α-keto glutaric	Lactic	Oxalo-acetic	Succinic	Tartaric
rom soil									
Saccharomyces delbrueckii	+	+	−	+	−	+	±	−	−
Cryptococcus albidus	+	+	+	+	+	−	−	+	+
C. diffluens	+	+	+	+	+	−	−	+	±
C. terreus	+	+	+	+	+	−	−	+	±
Candida tropicalis	+	+	+	+	+	+	−	+	−
C. humicola	+	+	+	+	+	+	+	+	+
C. parapsilosis	+	+	+	+	+	+	+	+	−
C. curvata	+	+	+	+	+	+	+	+	+
C. muscorum	+	+	+	+	+	+	−	+	+
Trichosporon pullulans	+	+	+	+	+	+	−	+	+
T. cutaneum	+	±	±	±	±	+	±	+	±
rom phyllosphere									
Sporobolomyces roseus	+	+	+	+	+	+	+	+	−
Cryptococcus laurentii	+	+	+	±	+	−	−	+	+
Torulopsis ingeniosa	+	+	+	+	+	+	+	+	−
Rhodotorula glutinis	+	+	+	+	+	+	+	+	+
R. mucilaginosa	+	+	+	+	+	+	+	+	+
R. graminis	+	+	+	+	+	+	+	+	−
iscellaneous									
Debaryomyces hansenii	+	+	+	+	+	+	+	+	−
D. kloeckeri	+	+	+	+	+	+	+	+	−
Sporobolomyces pararoseus	+	+	+	+	+	+	+	+	−
Cryptococcus luteolus	+	+	+	+	+	−	+	+	−
Candida guilliermondii	+	+	+	+	+	+	+	+	−
Schizoblastosporion starkeyi-henricii	±	−	+	+	+	−	−	+	−

± = Reaction varies with isolate.

Amylolytic activity. None of the fermenting yeasts used all the soluble starch in the medium, but a large proportion of the other soil and leaf yeasts caused its complete disappearance. Amylolytic activity was not always correlated with starch synthesis, neither did the reverse necessarily apply.

Effect of decrease in concentration of nutrients

Carbon and nitrogen sources. The method used was one which could be expected to reflect only gross differences in demand for these nutrients; no such differences were seen. After 2 weeks of incubation all species tested showed as abundant growth on solid auxanographic medium with 0·2 and

0·05 % ammonium sulphate as upon the standard with 0·5 %. Growth upon the medium with 0·01 % ammonium sulphate was only a little decreased, and was still considerable with 0·0025 % (25 p.p.m.) of ammonium sulphate added, but the agar used to solidify the medium added a further 4 p.p.m. (Dr D. J. Ross, personal communication).

Table 3. *Some nutritional characters of yeasts*

The nutritional rating is the sum of tests positive in Tables 2 and 3. Starch synthesis and use of nitrate nitrogen are not include in the rating.

	Use of				Degradation of						
	Ferulic acid	*p*-hydroxy-benzal-dehyde	Vanillin	Cello-biose	Gelatin	Pectin	Soluble starch	Starch synthesis	Synthesis of growth factors	Use of nitrate nitrogen	Nutri-tional rating
From soil											
Saccharomyces delbrueckii	–	–	–	–	–	–	–	–	–	–	4½
Cryptococcus albidus	+	–	–	+	–	–	+	+	+	+	12
C. diffluens	±	–	–	+	±	–	+	+	+	+	10½
C. terreus	+	+	–	+	–	–	+	+	+	+	11
Candida tropicalis	–	–	–	+	–	–	–	–	+	–	9
C. humicola	–	+	–	+	–	+	–	+	–	–	12
C. parapsilosis	–	–	–	+	–	–	–	–	–	–	9
C. curvata	–	–	–	+	–	–	+	+	–	–	11
C. muscorum	+	+	+	+	+	–	+	–	+	+	15
Trichosporon pullulans	+	+	+	+	+	–	+	+	+	+	15
T. cutaneum	–	+	–	+	+	–	–	+	–	–	8
From phyllosphere											
Sporobolomyces roseus	+	+	+	+	+	–	+	–	+	+	15
Cryptococcus laurentii	–	–	–	+	–	–	+	+	–	–	8½
Torulopsis ingeniosa	+	+	+	+	+	+	+	–	+	+	16
Rhodotorula glutinis	+	+	+	+	–	–	+	–	+	+	15
R. mucilaginosa	+	+	±	+	–	–	–	–	–	–	12½
R. graminis	+	+	+	+	±	–	+	–	+	+	14½
Miscellaneous											
Debaryomyces hansenii	–	–	–	+	–	–	–	–	–	–	9
D. kloeckeri	–	–	–	+	–	–	–	–	–	–	9
Sporobolomyces pararoseus	+	+	+	+	–	–	+	–	+	–	14
Cryptococcus luteolus	–	–	–	+	–	–	–	+	–	–	8
Candida guilliermondii	–	–	–	+	–	–	–	–	–	–	9
Schizoblastosporion starkeyi-henricii	–	–	–	–	–	–	–	–	–	–	4½

±=Reaction varies with isolate.

Decrease in concentration of glucose in the auxanographic medium was followed by a more rapid decrease in growth, which again did not seem to vary among the species tested. After 2 weeks of incubation growth was detectably less at a glucose concentration of 0·1 % than at the standard 0·5 %. There was a further decrease in the amount of growth at a concentration of 0·05 % and only weak growth at 0·01 % (100 p.p.m.). With no added available carbon source growth was lacking or only just detectable.

Mineral nutrients (Table 4). Results from this set of experiments are difficult to interpret. Almost all isolates showed some drop in amount of growth when the concentration of the mixture of mineral nutrients was decreased to 1/10 standard. It seems that under the conditions in which the cultures were grown, the standard concentration of one or several of the minerals supplied was optimal or sub-optimal in respect to the proportions of carbon, nitrogen and growth factors. Although there was tolerably good agreement in reaction between different isolates of some species, other species showed such variability between isolates that interspecific comparisons do not seem justifiable.

Part of the variations may have been due to authentic isolate differences in demand for minerals, part to mineral contamination of the cultures. A good deal of the variability seemed due to differences in amount of surface growth and consequent differences in oxygen supply. Use of culture vessels with a larger ratio of surface area: volume might overcome part of this, but more consistent results would probably be obtained by use of shaken cultures (Gavin, 1957).

Table 4. *Effect upon growth of decrease of mineral nutrients in the defined medium*

Figures in column headed 'standard' are drum readings from a Spekker photoelectric absorptiometer; figures in other columns are drum readings expressed as a percentage of the reading under 'standard'. A cell 0·25 cm. deep was used for readings from cultures in media with standard and 1/10 standard mineral nutrients. A 1 cm. cell was used for readings from the less dense cultures in media with 1/100, 1/1000 and no mineral nutrients; these were divided by four before calculating the percentage figures shown.

	Concentration of mineral nutrients				
From soil	Standard	1/10 (%)	1/100 (%)	1/1000 (%)	None (%)
Saccharomyces delbrueckii	0·475	50	4	1	1
	0·480	49	8	1	1
Cryptococcus albidus	0·233	92	79	49	15
	0·231	—	74	33	3
C. diffluens	0·133	57	48	23	4
C. terreus	0·182	104	62	10	4
	0·210	71	56	19	2
Candida tropicalis	0·699	94	30	12	6
C. humicola	0·450	82	55	25	4
	0·538	65	34	18	4
C. curvata	0·281	95	67	30	8
	0·342	75	53	33	14
C. muscorum	0·883	117	19	11	8
	0·957	99	22	9	6
Trichosporon pullulans	0·188	70	63	15	4
T. cutaneum	0·247	48	36	9	2
	0·237	76	43	13	3
From phyllosphere					
Cryptococcus laurentii	0·420	51	26	8	1
	0·210	85	65	25	2
Torulopsis ingeniosa	0·278	98	62	21	2
	0·437	56	40	2	1
Rhodotorula glutinis	0·201	83	78	12	2
	0·490	73	53	23	7
R. mucilaginosa	0·204	73	67	36	15
R. graminis	0·752	92	23	13	2
	0·769	89	24	10	1
Miscellaneous					
Debaryomyces hansenii	0·250	80	62	16	2
D. kloeckeri	0·224	73	52	16	2
	0·272	93	49	23	4
Sporobolomyces pararoseus	0·712	87	17	9	1
Cryptococcus luteolus	0·198	89	70	42	3
Candida guilliermondii	0·418	86	45	22	1
	0·772	73	22	11	—
Schizoblastosporion starkeyi-henricii	0·156	83	12	6	6
	0·100	81	10	1	5

Growth factors. There were differences in response to decreased concentrations of the standard growth factor mixture. All isolates produce as dense growth at 1/10 standard concentration as at standard. Some species, none of which could use nitrate nitrogen, grew only slightly when the concentration was decreased to 1/1000, and very slightly or not at all with no added growth factors. Of the species which could not use nitrate nitrogen, only *Sporobolomyces pararoseus* and *Candida tropicalis* were able to synthesize growth factors. All species which could assimilate nitrate nitrogen were able to grow without added growth factors except for one isolate of *Rhodotorula glutinis*. This isolate was aberrant in other ways, being unable to use any of the aromatic compounds tested as carbon sources, nor could it use tartaric acid, but it survived desiccation for 20 weeks, whilst the other isolate used was viable for only 8 weeks. It did not seem to be a commonly occurring variant, for three additional isolates tested used the aromatic compounds and synthesized growth factors, four additional isolates assimilated tartaric acid, and of a total of eight isolates, all of which could split soluble starch, it was the only one which could also synthesize a starch on Mager & Aschner's medium. Skinner & Huxley (1956), who noted a correlation between use of nitrate nitrogen and synthesis of growth factors, reported that all the five isolates of *R. glutinis* which they used grew in a vitamin-free medium.

DISCUSSION

In an attempt to summarize the results of the nutritional tests, the species examined have been given ratings (Table 3); one point was given for each test positive in Tables 2 and 3, except for use of nitrate nitrogen and for starch synthesis, and the sum of the points was the rating. Where a reaction was variable half a point was given, and where all species tested gave a positive result no points were given. It is realized that the tests applied were of unequal significance; ability to synthesize growth factors doubtless confers greater advantage than ability to assimilate one of a series of organic acids, but it seems impossible to assess the value of the characters more precisely.

On the whole the species isolated consistently as dominants (i.e. dominant yeasts) from the soil and from the phyllosphere of pasture plants, had higher ratings than those included in the miscellaneous group, species which have not yet been found as dominants on vegetation or in soil. Almost all the species which could synthesize growth factors and assimilate aromatic compounds, and which had amylolytic, pectinolytic or proteolytic powers, are included in the soil and phyllosphere groups.

The work done has shown that the yeasts which are common in the soil and the phyllosphere are equipped to use foodstuffs which may occur in these habitats. Some other species, less common in these places, but still numerous enough to appear relatively frequently in population samples, have lesser catabolic powers, as indicated by lower ratings. Except for those species which can degrade pectin, it does not seem likely that soil and phyllosphere yeasts play a primary part in the breakdown of vegetable matter, but rather that they follow organisms which attack cellulose and lignin and that they use the resulting degradation products.

The tests applied show no consistent differences between soil and phyllosphere species, yet in the field these two groups are distinct. In surveys made so far soil yeasts have rarely been found upon the leaves of pasture plants, and although phyllosphere yeasts do invade the upper layers of the soil, particularly in summer when numbers on leaves are very high, they do not persist. It is possible that the wrong techniques have been used to show why one species can and does live in soil and another on leaves. One of the limitations of this work is the lack of precise information about the foodstuffs present and available in soil and on plant leaves. Garrett (1957) has written: 'The complex interlocking social structure of most natural communities is determined first by the selective action of the habitat and secondly by the interactions of its inhabitants.' The selective action of the habitats, a rather low but perhaps fairly constant concentration of somewhat complex foodstuffs, would explain the physiological similarities between soil and leaf yeasts; the interactions of the inhabitants may explain why the two yeast populations are distinct.

Soil Bureau Publication No. 143.

REFERENCES

BELL, T. A. & ETCHELLS, J. L. (1956). Pectin hydrolysis by certain salt-tolerant yeasts. *Appl. Microbiol.* **4**, 196.

GARRETT, S. D. (1957). Ecologists in the laboratory. *Nature, Lond.* **180**, 351.

GAVIN, J. J. (1957). Analytical microbiology. III. Turbidometric methods. *Appl. Microbiol.* **5**, 235.

KURUNG, J. N. (1942). The isolation and identification of pathogenic fungi from sputum. *Amer. Rev. Tuberc.* **46**, 365.

LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts. A Taxonomic Study.* Amsterdam: North Holland Publishing Co.

MAGER, J. & ASCHNER, M. (1947). Biological studies on capsulated yeasts. *J. Bact.* **53**, 283.

MENNA, M. E. DI (1954). Non-pathogenic yeasts of the human skin and alimentary tract. *J. Path. Bact.* **68**, 89.

MENNA, M. E. DI (1955*a*). A search for pathogenic species of yeasts in New Zealand soils. *J. gen. Microbiol.* **12**, 54.

MENNA, M. E. DI (1955*b*). A quantitative study of air-borne fungus spores in Dunedin, New Zealand. *Trans. Brit. mycol. Soc.* **38**, 119.

MENNA, M. E. DI (1957). The isolation of yeasts from soil. *J. gen. Microbiol.* **17**, 678.

MENNA, M. E. DI (1958). Biological studies of some tussock grassland soils. 3. Yeasts of three tussock grassland soils. *N.Z. J. Agric. Res.* **1**, 939.

SKINNER, C. E. & HUXLEY, M. J. (1956). *Rhodotorula glutinis. Mycologia*, **48**, 371.

UDEN, N. VAN & DO CARMO SOUSA, L. (1957). Yeasts from the bovine caecum. *J. gen. Microbiol.* **16**, 385.

WIERINGA, K. T. (1955). Der Abbau der Pektine; der erste Angriff der organischen pflanzensubstanz. *Z. Pflernähr. Düng.* **69**, 150.

WIERINGA, K. T. (1956). The micro-organisms decomposing pectic substances in the dew retting process of flax. *Neth. J. agri. Sci.* **4**, 204.

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Kocková-Kratochvílová, A. & Svobodová-Poláková, Y. (1959). *J. gen. Microbiol.* **20**, 24–26

Nadsonia slovaca n.sp. Isolated in Slovakia

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SUMMARY: In the region of the mountains of Malé Karpaty and Harmanec in Slovakia a new species, *Nadsonia slovaca*, was isolated. The species was found on the surface of the fruits of *Cornus mas*, on the surface of the fruiting bodies of *Amanita muscaria*, *Nematoloma lateritium* and *N. fasciculare* and in the water of the brook Bystrica. The new species differed from those described hitherto by the formation of abundant pseudomycelium on the margin of slimy streaks and by the formation of big, bag-like, granulated cells. The new species did not ferment any sugars.

In 1957 we studied the natural yeast microflora in the region of Malé Karpaty and in the water of the brook Bystrica in the neighbourhood of Harmanec. We isolated several strains of the genus *Nadsonia*. The isolation was accompanied with considerable difficulties as all these strains were contaminated by bacteria which resisted the acidic nature of Raulin's solution. We succeeded in isolating six strains: MK39 from the surface of the fruits of *Cornus mas*, growing in a hedge of vineyard on the slopes of the Malé Karpaty mountains near Modra; MK15 from the surface of *Amanita muscaria*, growing in a pine forest in the vicinity of a deserted stone quarry, MK42 from the surface of *Nematoloma lateritium* growing in a mixed oak-beech forest along the road from Červený Kameň to a locality called Karlubek in the mountains of Malé Karpaty; MK43 from the surface of *N. fasciculare* growing in a mixed oak-beech forest in a locality called Karlubek, which is north-west of the castle of Červený Kameň. Strains H11 (H1V) and H12 (H2V) were isolated from the water of the brook Bystrica, the water of which was contaminated by waste water from a paper factory. The brook Bystrica was divided into five profiles of different intensity of pollution and both strains were isolated from the profile no. 4 belonging to the polysaprobic biological region with a tendency to alfa-mesosaproby. The temperature of the water of the brook Bystrica at the time of sampling was 14·5°. The strains were isolated from water and from natural sources in August and September, respectively.

METHODS AND RESULTS

Growth in malt extract after 3 *days at* 25°. Pellicles were formed on the surface of the broth by all the cultures.

Dimensions of cells. See Table 1.

Growth on malt agar at 25° *after* 3 *days.* The brownish ivory-coloured growth was slimy, glistening and high; it formed abundant pseudomycelium on the margin. Upon prolonged cultivation on wort agar the slimy character diminished and acquired a fatty glistening and wrinkled appearance with a still more abundant growth of pseudomycelium.

Slide cultures on potato agar at 25°. A distinct pseudomycelium was formed on the margin of the colonies after 5 days of cultivation (Pl. 1, fig. 1). The pseudomycelium formed by cells of similar morphological character was irregularly branched.

Table 1. *Dimensions of cells of the six strains of* Nadsonia slovaca, *n.sp.*

Strain no.	Size variances (μ)	Mean length (μ)		Mean width (μ)		r coefficient of correlation	l:w ratio of length and width
		$\bar{x}$	$s\bar{x}$	$\bar{y}$	$s\bar{y}$		
MK15	(4–12) × (2– 5)	6·46	0·192	2·86	0·083	0·16	2·25
MK39	(2– 9) × (2– 8)	7·46	0·183	3·98	0·092	0·27	1·87
MK42	(2– 9) × (2– 8)	7·01	0·164	3·77	0·086	0·41	1·85
MK43	(3–16) × (2– 5)	6·98	0·217	3·45	0·073	0·46	2·02
H1V	(3– 6) × (4–10)	6·72	0·107	4·24	0·021	0·45	1·57
H2V	(3–16) × (4–10)	8·15	0·173	5·02	0·068	0·51	1·62

Sporulation. The spores of all six strains were observed in a phase contrast microscope. The sporulation of our strains was similar to the sporulation of the genus *Nadsonia*, described in the literature (Lodder & Kreger-van Rij, 1952). The spores appeared big, spherical, white with a bright brownish-red centre. Before sporulation large, bag-like cells with granules possessing high refractive power were observed (Pl. 1, fig. 2). We consider the bag-like cells to be in a presporulative state, which was observed by us also with other sporulating yeasts (Kocková-Kratochvílová, Kutková & Petrová, 1957). Usually one spore was seen in every ascus.

Assimilation of individual sugars. All six strains assimilated glucose, galactose, maltose, sucrose, lactose and arabinose.

Assimilation of sugars from a solution containing different sugars. (Kocková-Kratochvílová & Vojtková-Lepšíková, 1958). From a solution containing different sugars all six strains assimilated arabinose and galactose prior to the assimilation of maltose.

Optimal cultivation temperature. 18–20°.

Fermentation of sugars. Absent.

Assimilation of potassium nitrate. Positive.

Assimilation of ethanol. Ethanol was assimilated extremely well by all six strains, which formed a thick pellicle on the surface of the medium.

DISCUSSION

Our description was made according to the procedure of Lodder & Kreger-van Rij (1952) and according to Kudriavtshev (1954). As Lodder & Kreger-van Rij did not describe any pseudomycelium-forming species of the genus *Nadsonia* we originally presumed our strains to be similar to the genus *Nadsoniomyces* Kudriavtshev. We tried to compare the bag-like granulated cells of our strains with the cells of *Nadsoniomyces*, which are pointed at one end. The granules of our bag-like cells possess no filaments nor did we observe any formation of arthrospores, both of which are described by Kudriavtshev. As our strains resemble the genus *Nadsonia* Sydow, we have decided to include our strains in this genus. Our strains differed only by the formation of abundant pseudomycelium and by the absence of fermentation of sugars. We consider our strains to be a new species, which we name *Nadsonia slovaca*. It has been deposited in the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Latin diagnosis. Nadsonia slovaca sp.n.

In musto maltato cellulae oblongae, rhomboidae, praeacutae, (2–16) × (2–10) μ, singulae. Post dies 3 pellicula crispulata, flavalbida, atque sedimentum formantur.

In agare maltato cellulae formae et dimensiones, cellularum eadem sunt, qua in musto maltato. Cultura post dies 15 flavalbida, glabra et mucilaginosa, margine piloso. Pseudomycelium abundant. Cellulae pseudomycelii oblongae in catenis ramosis.

Ascosporae rotundae, magnae, albae cum centro rubrobruneo. Sporulatio talis, qualis apud genem Nadsonia describitur.

Fermentatio nulla. In medio minerali cum glucoso, maltoso, saccharo, lactoso, arabinoso crescit. Nitras Kalii assimilatur. In medio minerali cum alcohole aethylico crescit, magna pellicula formatur.

REFERENCES

Kocková-Kratochvílová, A., Kutková, M. & Petrová, M. (1957). Zur Frage des Erregers der interstitiellen plasmazellulären Pneumonie bei Säuglingen. *Naturwissenschaften*, **44**, 565.

Kocková-Kratochvílová, A. & Vojtková-Lepšíková, A. (1958). Eine neue Art der Beurteilung der Assimilationsfähigkeit der Hefe. *Naturwissenschaften*, **45**, 773.

Kudriavtshev, V. I. (1954). *Sistematika drozhej.* Moskva: Izdatelstvo Akademii Nauk SSSR.

Lodder, J. & Kreger-van Rij, N. J. W. (1952). *The Yeasts.* Amsterdam: North Holland Publishing Company.

EXPLANATION OF PLATE

Fig. 1. Marginal mycelium on a slide culture on potato agar.
Fig. 2. Cells of *Nadsonia slovaca* n.sp., bag-like cells and germinating spores. × 1000.

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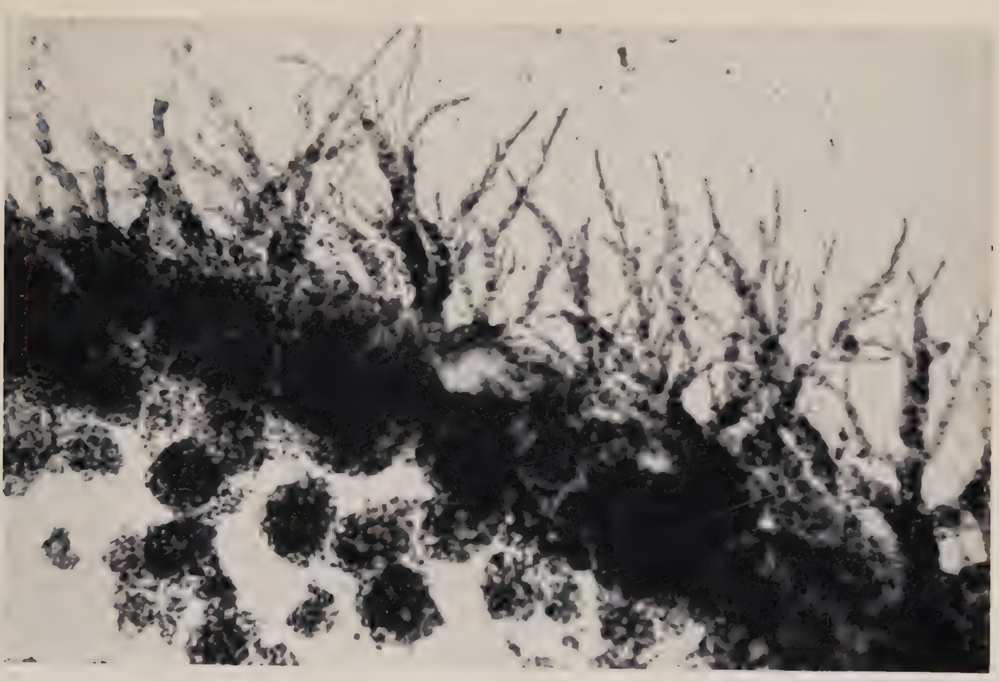

Fig. 1

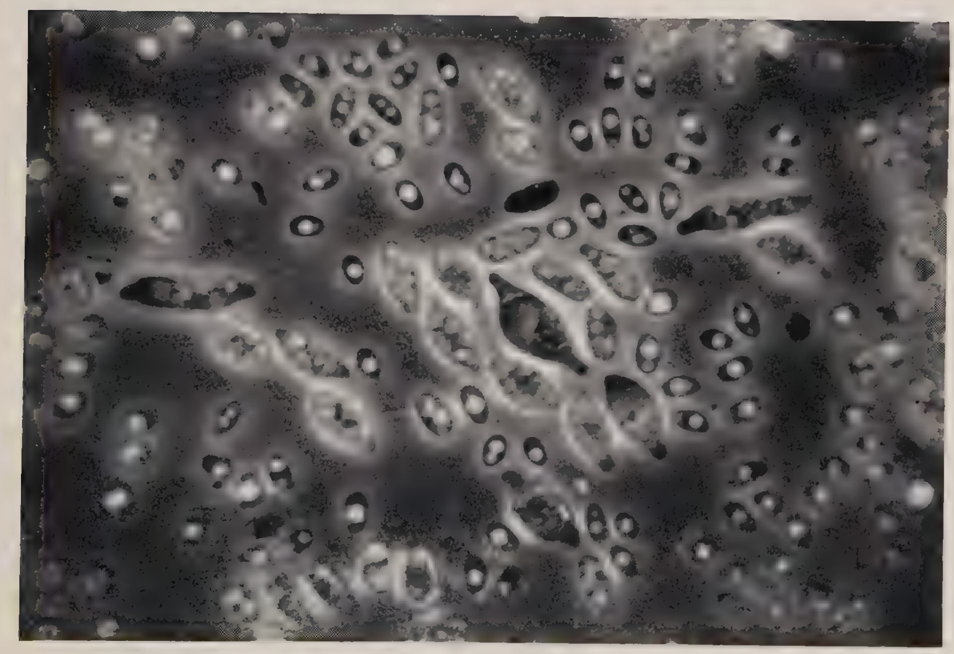

Fig. 2

A. Kocková-Kratochvílová & Y. Svobodová-Poláková—*Nadsonia slovaca* n.sp.
Plate 1

(*Facing p.* 26)

Christian, J. H. B. & Ingram, M. (1959). *J. gen. Microbiol.* **20**, 27–31

The Freezing Points of Bacterial Cells in Relation to Halophilism

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SUMMARY: The hypothesis that halophilic bacteria achieve a high degree of salt tolerance by exclusion of much of the external solutes of the growth medium has been tested. Organisms were grown in media containing from 1 to 4 M salts. For both halophilic and non-halophilic bacteria the freezing points of the cells were close to those of the media in which they were grown. It is concluded that halophilic bacteria do not maintain the intracellular aqueous phase at a water activity greater than in the surrounding growth medium.

One possible explanation for the phenomenon of bacterial halophilism is that the solutes in the aqueous phase within halophilic cells are much less concentrated than in the surrounding medium (Robinson, Gibbons & Thatcher, 1952). Such a system might be maintained, apart from mechanisms of accumulation and excretion, by a membrane impermeable to external solutes and a rigid cell boundary which resists plasmolysis. To examine this hypothesis, the freezing-point depressions of bacterial suspensions and of their growth media have been determined and compared. The bacteria included both halophilic and non-halophilic strains grown in media containing various concentrations of salts. No appreciable differences in the osmotic status of these two types of bacteria were observed.

METHODS

The four halophilic strains tested were *Micrococcus halodenitrificans*, *Vibrio costicolus*, *Halobacterium halobium* and *Sarcina littoralis* from the collection of Dr N. E. Gibbons, National Research Council, Ottowa. Other cultures used were *Micrococcus lysodeikticus* (NCTC 2665), *Staphylococcus aureus*, *Escherichia coli* and a salt-tolerant *Bacillus circulans* from the collection of the Low Temperature Research Station, Cambridge.

The red halophiles (*Halobacterium halobium* and *Sarcina littoralis*) were grown on the medium of Baxter & Gibbons (1954), but with 2 M-NaCl and 2 M-KCl. For all other bacteria the growth medium contained 0·5 % (w/v) each of proteose peptone (Difco) and tryptone (Difco) with additions of NaCl, or of NaCl and KCl as required. The initial pH value was 7·0 in all media. These media were solidified by 2 % (w/v) Bacto agar.

To decrease the amount of contaminating medium in suspensions of organisms, the bacteria were grown on solid media in Petri dishes. An inoculum of 0·1 ml. was spread evenly over the surface. The plates were dried at the temperature of subsequent incubation until no free liquid remained on the surface. They were incubated inverted and without lids in sealed desiccators over salt solutions isotonic with the growth medium. The incubation temperature was 37° for red halophiles and 30° for other strains. The incubation period varied with the organism and with the concentration of salts.

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The organisms so produced were carefully scraped from the medium by means of a thick wire and rapidly transferred to a tapered glass centrifuge tube, which was stoppered between transfers to limit evaporation. After weighing, the stoppered tube and its contents were autoclaved to break down the cell permeability barriers and any water lost was replaced from a fine capillary pipette. The wet weight of the samples ranged from 0·35 to 1·7 g.

Freezing points were determined with a copper-constantan thermocouple, and the temperature read directly from a Pye Scalamp galvanometer calibrated in degrees Centigrade. The tip of the thermocouple was wound into a tight spiral with the junction bent inwards, so that when inserted into the cell debris the junction was centrally situated away from the wall of the tube. The thermocouple leads were held in position by a cork stopper. In most experiments the freezing mixture was NaCl + ice, but for determinations with the red halophiles $CaCl_2$ + ice mixtures were necessary to give initial temperatures near −30°.

RESULTS

The initial freezing of the sample at a cooling rate of about 2°/min. involved substantial supercooling and the resulting freezing point was spuriously low. It was plainly essential to avoid such error. Accordingly, the sample was thawed and replaced in a freezing-mixture which gave a lower rate of freezing, so that subsequent supercooling was less and the freezing-point determination more accurate; this procedure was repeated, until the freezing point became constant. Values thus determined both during freezing and subsequent thawing never differed by more than 0·1°, indicating that supercooling was not occurring.

Because, as mentioned above, it was frequently necessary to restore water lost from the pellet of cells during the previous heat treatment, freezing points were recorded with the thermocouple in three different positions. When the three values differed by more than 0·1°, it was concluded that the solutes were imperfectly distributed in the aqueous phase, so the preparation was stirred with the thermocouple, and the observations repeated. The quoted values are the averages of the last three determinations.

Freezing points were similarly determined for all media before addition of agar and for all equilibrating salt solutions after growth. These two values agreed closely. Scott (1953) stated that the effect of the usual concentrations of agar on the water activity of a medium is very small.

The relationship between the freezing points of heated cell preparations and freezing points of the growth media are shown in Fig. 1, where the points refer to media with aggregate salt concentrations of 1, 2 or 4 M. For a given growth medium the values obtained with non-halophilic bacteria were generally slightly lower than those found for the moderate halophiles. However, within both groups the differences between species were too large for any significance to be attached to this observation. There were no differences between organisms grown on media containing additions of NaCl and KCl or NaCl alone.

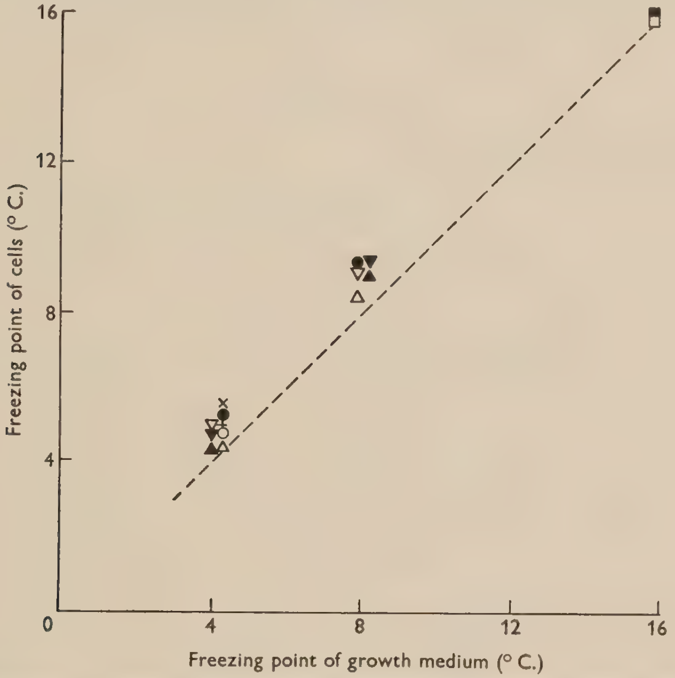

Fig. 1. **Relationship between freezing points of heated organisms and growth medium. The broken line is isothermal. From media containing added NaCl only: ▲, *Micrococcus halodenitrificans*; ▼, *Vibrio costicolus*. From media containing equimolar additions of NaCl and KCl: ×, *Staphylococcus aureus*; △, *Micrococcus halodenitrificans*; +, *Escherichia coli*; ▽, *Vibrio costicolus*; ○, *Micrococcus lysodeikticus*; □, *Halobacterium halobium*; ●, *Bacillus circulans*; ■, *Sarcina littoralis*.**

The amount of contaminating growth medium contributing to the freezing point of the samples is not known. However, since the freezing points of all organisms except *Halobacterium halobium* were lower than those of their growth medium, corrections for contamination would increase this deficit. Hence, the freezing points of the halophilic and non-halophilic organisms were equal to or lower than the freezing points of the media on which they were grown.

DISCUSSION

The use of cryoscopic data to determine the internal osmotic status of cells is open to criticism. A major objection is that in disrupting the cells considerable changes may occur in the distribution and association of intracellular substances. Thus constituents which are normally combined in an osmotically inactive form might be liberated and large complexes broken down to smaller, more osmotically active units. This criticism is largely answered by the data of Mitchell & Moyle (1956) and of Scott (1953). Mitchell & Moyle estimated the osmotic pressure within *Staphylococcus aureus* by two independent methods. By equilibrating washed organisms over sucrose solutions of known concentration they deduced an osmotic pressure of 20–25 atmospheres. From the weight of solutes extracted by trichloroacetic acid from organisms of known water content they calculated a value of 20–30 atmospheres. Scott obtained similar results by the cryoscopic method. From the freezing-point depression of unwashed, centrifuged and heated *S. aureus* an osmotic pressure of about 25 atmospheres was obtained after allowing for the contribution of contaminating growth medium.

The results in Fig. 1 show that in no case was the internal aqueous content of the halophilic or non-halophilic bacteria tested likely to be less concentrated than the growth medium. Hence, it is concluded that halophilic bacteria do not owe their salt tolerant properties to an ability to maintain a comparatively low internal solute concentration.

It is further interesting that no difference appeared in pattern of behaviour between the less extreme halophiles like *Micrococcus halodenitrificans* and the most extreme red types like *Sarcina littoralis* or *Halobacterium halobium*. Gibbons & Baxter (1953) concluded that the internal concentration of sodium chloride in *Micrococcus halodenitrificans* remained at 5 % in organisms grown in concentrations ranging from 8 to 22 %, whereas the concentration in *Sarcina littoralis* increased linearly from 10 to 19 % with increase of external concentration from 18 to 27 %. This encouraged Baxter & Gibbons (1954) to suggest that the group of organisms typified by *Micrococcus halodenitrificans* possesses a special mechanism, absent from the red group, for maintaining the intracellular concentration well below that of the environment. The apparent implication would be that, with organisms grown in about 4M-NaCl, their freezing point should be about $-5°$ for *M. halodenitrificans* but about $-10°$, roughly twice as great, for *Sarcina littoralis*. Our experiments, besides indicating freezing points lower than the above values, give no hint of any such difference in behaviour between the two groups of organisms. Because the conclusion of Gibbons & Baxter was based on estimations of chloride, however, the differences might well be due to the existence of additional solutes not accounted for by means of chloride estimations.

The nature and contribution of all the internal solutes is not known but Ingram (1938), and Yamada & Shiio (1953), indicated that there is substantial excess of Na^+ over Cl^-; and Christian (1956) found that halophilic organisms contained large amounts of potassium as well as sodium, the former probably predominating in the red extreme halophiles. Thus it is suggested that the salts of both these cations are largely responsible for the low internal water activities indicated by freezing-point determinations.

For a given strain grown in widely differing concentrations of salts there was a fairly constant difference between the freezing points of the cell contents and the growth medium. It has also been shown (Christian & Ingram, 1959) that the osmotic sensitivity of both halophilic and non-halophilic bacteria is proportional to the NaCl content of the medium during growth. From these data it is concluded that the osmotic status of bacterial cells is very largely determined by the water activity of the growth medium.

This work was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research during the tenure by one of us (J.H.B.S.) of an Overseas Studentship of the Commonwealth Scientific and Industrial Research Organization.

REFERENCES

Baxter, R. M. & Gibbons, N. E. (1954). The glycerol dehydrogenases of *Pseudomonas salinaria*, *Vibrio costicolus* and *Escherichia coli* in relation to bacterial halophilism. *Canad. J. Biochem. Physiol.* **32**, 206.

Christian, J. H. B. (1956). The physiological basis of salt tolerance in halophilic bacteria. Dissertation, Cambridge.

Christian, J. H. B. & Ingram, M. (1959). Lysis of *Vibrio costicolus* by osmotic shock. *J. gen. Microbiol.* **20**, 32.

Gibbons, N. E. & Baxter, R. M. (1953). The relation between salt concentration and enzyme activity in halophilic bacteria. *Proc. VIth int. Congr. Microbiol.*, Rome, **1**, 210.

Ingram, M. (1938). The effect of sodium chloride on a bacterial enzyme which destroys lactic acid. *Rep. Fd Invest. Bd Lond.*, p. 72.

Mitchell, P. & Moyle, J. (1956). Osmotic structure and function in bacteria. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 150.

Robinson, J., Gibbons, N. E. & Thatcher, F. S. (1952). A mechanism of halophilism in Micrococcus halodenitrificans. *J. Bact.* **64**, 69.

Scott, W. J. (1953). Water relations of *Staphylococcus aureus* at 30° C. *Aust. J. biol. Sci.* **6**, 549.

Yamada, T. & Shiio, I. (1953). Effects of salt concentration on the respiration of a halotolerant bacterium. *J. Biochem., Tokyo*, **40**, 327.

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Lysis of *Vibrio costicolus* by Osmotic Shock

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SUMMARY: Suspensions of the halophilic organism *Vibrio costicolus* lysed when subjected to sufficient osmotic shock; this was revealed as a marked fall in optical density. Organisms in the logarithmic phase of growth, grown in media containing M-NaCl, when suspended in NaCl or LiCl solutions with concentrations M or lower lysed when the concentration was below 0·33 M: when the organisms were suspended similarly in KCl or NH_4Cl solutions, lysis began at a concentration above 0·8 M. Older organisms were more resistant to lysis. Lysis was little affected by replacement of chloride by other univalent anions, by pH values from 4 to 10 or by previous incubation with 10^{-5} M-mercuric chloride. Addition of cetyltrimethylammonium bromide (200 μg./ml.) increased resistance to lysis in both NaCl and KCl solutions.

Organisms grown in media of NaCl concentrations ranging from 0·5 to 3·0 M began to lyse at a NaCl concentration one-third of that present during growth. Organisms suspended in NaCl solutions of one-half the concentration in which they had been grown equilibrated promptly to the new environment; this was shown by a decrease of 50 % in the salt concentration at which lysis then began. When organisms were transferred to salt concentrations higher than that of the growth medium, the osmotic sensitivity was not affected. It is concluded that the osmotic status of these organisms is controlled chiefly by small ions or molecules which leak rapidly from the cells on transfer to dilute environments.

Many types of bacteria lyse when transferred from concentrated growth media to more dilute solutions or to water, and this behaviour is particularly common among halophilic strains. Robinson & Katznelson (1953) noted that the extremely halophilic *Pseudomonas salinaria*, grown in a medium containing 16 % (w/v) NaCl, underwent rapid plasmoptysis on transfer to 1, 2 or 4 % NaCl and showed some deterioration of structure in 8 % NaCl. However, no attempts appear to have been made to study quantitatively the degree of lysis resulting from osmotic shock. Christian & Ingram (1959) found that the freezing-point depression of heated bacteria was similar to that of their growth medium over a wide range of salt concentrations. It was of interest, therefore, to investigate the relationship between the osmotic sensitivity of such organisms and the salt concentration of the environment. This paper describes a technique for measurement of osmotic lysis, and reports the effects of a number of factors on the osmotic sensitivity of a moderate halophile, *Vibrio costicolus.*

METHODS

Organism. The test organism was a strain of *Vibrio costicolus* kindly supplied by Dr J. M. Shewan.

Media. The organism was grown in liquid media containing 0·5 % (w/v) each of proteose peptone (Difco) and tryptone (Difco) with additions of NaCl. The initial pH value was 7·0. Media for maintaining stock cultures and for growing inocula contained 4 % (w/v) NaCl. All cultures were incubated at 25°.

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Lysis technique. Cultures were grown in 200 ml. volumes of medium of appropriate NaCl content, aerated by shaking, in Fernbach flasks. Organisms were harvested in the logarithmic phase of growth and a thick suspension prepared in a solution of the appropriate salt. Except where otherwise stated, this solution was equimolar with the salt concentration used in the growth medium; and the concentration of organisms was chosen to give a reading about 30 on an E.E.L. colorimeter after tenfold dilution with the same salt solution. To produce osmotic shock, 1 ml. portions of the stock suspension were pipetted into ten 9 ml. volumes of graded concentrations of the same salt solution, and the mixtures stirred thoroughly. At least 2 min. were next allowed for equilibration and clearance of bubbles; beyond this, the time was not critical. The optical densities of the mixtures were measured in the E.E.L. colorimeter at a wavelength of about 450 mμ. These values are recorded graphically as percentage of the optical density of the suspension in the highest salt concentration tested. Variations in the NaCl content of the growth medium and in the nature and concentration of the salts used in the lysis experiments are described later.

RESULTS

For the sake of clarity, the main results are first presented in section A, assuming that changes in optical density correctly indicated the lysis of the cells, this assumption being justified later in section B.

A. *The influence of conditions on lysis*

The factors pH value and temperature proving relatively unimportant (see later), interest centred on the salt relations of the organisms.

Lysis in sodium chloride solutions. The relationship between NaCl concentration and optical density after osmotic shock for organisms grown in M-NaCl medium is shown in Fig. 1. The decrease in optical density, as the salt concentration was decreased from 1·0 to 0·7 M, was presumably due to osmotic swelling (cf. Mitchell & Moyle, 1956). The more rapid decrease at salt concentrations below 0·4 M was due to the lysis of an increasing proportion of the organisms, as will be shown later. At these low concentrations, the turbidity decreased rapidly as soon as the organisms were mixed with the diluting solutions and most of the time lag (< 2 min.) was due to slow clearing of air bubbles in the viscous lysate.

The shape of the curve in Fig. 1 is probably sigmoid, but the inflexion between the values 0·3 and 0·4 M is nevertheless well defined. To determine the salt concentration at which lysis might be said to begin, the two linear portions of the curve were extrapolated to intersect as shown in Fig. 1. By rough analogy with the terminology of plasmolysis, the salt concentration corresponding with this intersection has been termed the 'point of incipient lysis', or, simply, the 'lysis point'. Thus for logarithmic phase organisms, grown and suspended initially in M-NaCl, the lysis point was near 0·33 M. This value was obtained in most experiments, with occasional variations of 0·01 M. For organisms harvested in the stationary phase of growth, the lysis point was 0·2 M.

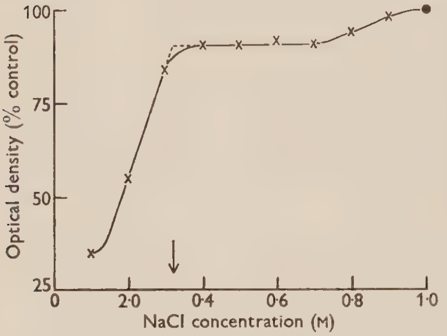

Fig. 1

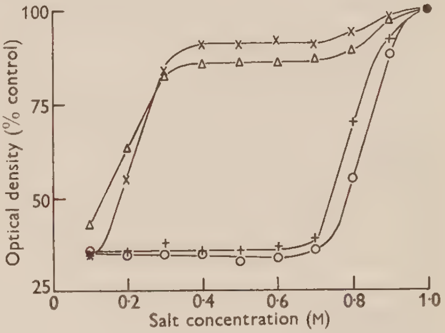

Fig. 2

Fig. 1. Effect of osmotic shock on the optical density of suspensions of *Vibrio costicolus*. Organisms were harvested in the logarithmic phase of growth from media containing M-NaCl. The arrow indicates the concentration at which lysis commences.

Fig. 2. Effect of osmotic shock in salt solutions on the optical density of suspensions of *Vibrio costicolus* ×, NaCl; △, LiCl; ○, KCl; +, NH_4Cl. Organisms were grown in media containing M-NaCl.

Lysis in solutions of other salts. Figure 2 shows the optical density curves obtained when organisms grown in medium containing M-NaCl were suspended in M solutions of LiCl, NaCl, KCl or NH_4Cl and subsequently diluted in the corresponding salt. In LiCl (and in $MgCl_2$) the results were very similar to those in NaCl. On the other hand, KCl and NH_4Cl afforded very little osmotic protection, and organisms began to lyse at concentrations near 0·9 M.

Other univalent anions (bromide, nitrate, thiocyanate, acetate) behaved similarly to chloride, with either sodium or potassium as cation. Sulphate was tested as a representative bivalent anion, giving twice the concentration of cations found in the corresponding uni-univalent salt solutions. In Na_2SO_4, typical lysis occurred at concentrations just below 0·2 M; i.e. the lysis point was at a Na^+ concentration between 0·3 and 0·4 M as in NaCl solutions. In K_2SO_4 the optical density began to fall at 0·8 M, but the subsequent decrease was less sharp than in KCl, so that the lysis point was ill-defined; it was, however, at a K^+ concentration near M, thus corresponding roughly with the situation in KCl represented in Fig. 2 (and Fig. 5). Because the lysis points are much the same in sulphate as in chloride solutions, when reckoned on cation concentration, while the great difference between sodium and potassium salts remains, the effects observed must be chiefly dependent on the nature of the cation.

Smithies, Gibbons & Bayley (1955) found that the addition of 0·15 % (w/v) $CaCl_2$ to the growth medium gave halophilic organisms from which improved preparations of cell envelope material could be obtained. Cell walls of such preparations were much more electron dense than those from organisms grown in normal medium. If these data indicate a strengthening of the cell wall by growth in $CaCl_2$, an enhanced resistance to lysis might be expected. However, *Vibrio costicolus* grown under these conditions behaved in the same way as organisms grown normally, when subjected to osmotic shock in either sodium or potassium chlorides.

Lysis of washed organisms. When *Vibrio costicolus* organisms were washed once in the solution in which they were finally to be suspended, they acquired a slightly increased resistance to osmotic shock in NaCl and in KCl. Since this change was variable and was probably due, at least in part, to ageing of the organisms, unwashed organisms were preferred for subsequent experiments.

Effect of temperature. The effect of temperature on lysis in sodium and potassium chloride solutions was examined by equilibrating duplicate batches of suspensions and salt solutions at 10° and 37°. Subsequent dilutions and optical density readings were carried out at these same temperatures. Lysis in KCl solution was not affected by temperature. In NaCl solutions, however, the osmotic sensitivity increased with increase in temperature. Lysis began at 0·28 M at 10° and at 0·36 M at 37°, compared with 0·33 M at room temperature. Suspensions which had been heated for 10 min. at 80° had low optical densities and did not give typical lysis curves when diluted.

Effect of pH value. All the lysis data given above refer to experiments in salt solutions without adjustment of pH value. To examine the effect of pH value on the reaction, organisms were diluted in : (i) unbuffered NaCl or KCl solutions adjusted to pH values over the range 4·0–10·0, with HCl and NaOH or KOH, respectively; (ii) in solutions adjusted to pH 5·5 and 8 with phosphate buffer (0·033 M–Na_2HPO_4/KH_2PO_4). In no case were the concentrations at which lysis began appreciably affected by the pH value or by the presence of the phosphate buffer.

Effect of antibacterial substances. $HgCl_2$ was found by Mager, Kuczynski, Schatzberg & Avi-Dor (1956) to inhibit the 'optical effect' which accompanied the transfer of bacteria to solutions of higher osmotic pressure. To test its effect on the lysis reaction, *Vibrio costicolus* organisms were incubated in 10^{-5} M-$HgCl_2$ for 15 min. before subjection to osmotic shock in solutions of NaCl and KCl. This treatment did not alter the optical density curves.

Robinson, Gibbons & Thatcher (1952) suggested that tetramethyl-*p*-phenylenediamine (TMPPD) might inhibit a system maintaining a differential in salt concentration across the cell wall of *Micrococcus halodenitrificans*. To examine the effect of TMPPD on lysis of *Vibrio costicolus*, 0·003 M-TMPPD was added to the suspensions 15 min. before diluting in NaCl and KCl solutions. In a few minutes the suspension became very viscous, apparently as the result of lysis. To avoid this, TMPPD was added to the salt solutions into which organisms were pipetted to produce osmotic shock. It was necessary to add ascorbic acid to decolorize the suspensions before measuring the optical density. Under these conditions TMPPD had little effect on the resulting lysis curves.

Cetyltrimethylammonium bromide (CTAB) is known to disorganize the cytoplasmic membrane of bacteria (Salton, 1951) and was tested for its effects on the osmotic lysis of *Vibrio costicolus*. CTAB was added to the stock suspension 2 min. before the dilution experiments began; at a concentration of 650 μg./ml. the organisms lysed rapidly and the stock suspension cleared. However, the suspension remained stable on addition of 200 μg. CTAB/ml. but when subjected to osmotic shock the lysis curves were abnormal, as shown in Fig. 3. In NaCl and in KCl solutions the treated organisms were much more resistant to osmotic shock.

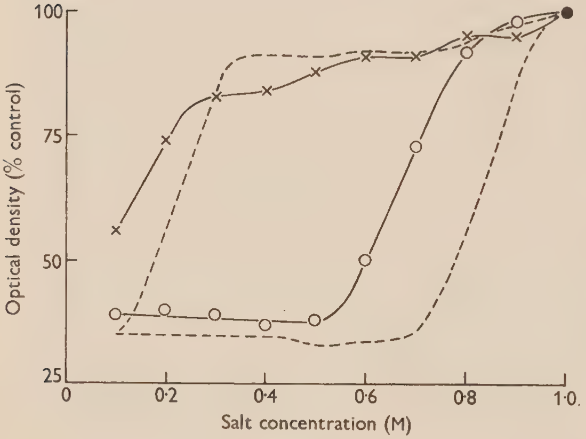

Fig. 3

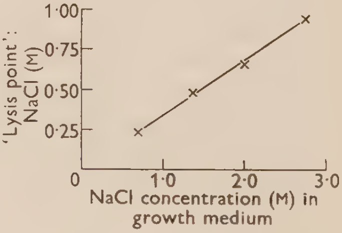

Fig. 4

Fig. 3. Effect of cetyltrimethylammonium bromide (200 μg./ml.) on lysis of *Vibrio costicolus*. ×, NaCl; ○, KCl. Continuous lines, with CTAB; broken lines, controls without CTAB, from Fig. 2. Organisms were grown in medium containing M-NaCl.

Fig. 4. Relationship between NaCl concentration at which lysis of *Vibrio costicolus* began and that in which the organisms were grown.

Influence of the sodium chloride content of the growth medium. For all experiments discussed so far, organisms were grown in media containing M-NaCl. When the growth medium contained 2·0 M-NaCl and the organisms were suspended in 2·0 M salt solutions before dilution to concentrations 2·0 to 0·2 M, the form of the curves in NaCl and in KCl was the same as found in the M system but all concentrations had been doubled and lysis began at twice the concentration found previously.

The lysis points were determined for logarithmic phase organisms grown in a range of salt concentrations, and are shown in Fig. 4. Within the limits of accuracy of this technique, the concentration of NaCl at which lysis began was about one-third of the salt concentration of the medium in which the organisms had been grown.

Effect of transfer to higher concentrations of salts. In all the experiments described above, organisms were suspended in salt solutions equimolar with the salt concentration of the growth medium and lysis began when the NaCl concentration was decreased to one-third of this. To determine the stability of this relationship, organisms grown in M-salt medium were suspended in 2·0 M solutions of NaCl or KCl before being subjected to osmotic shock. The results are shown in Fig. 5. Although the upper portions of both curves fall quite steeply as a result of changes in cell volume with concentration, the curves retain the points of inflexion relevant to the salt concentration in which they had grown. This is best seen in the curve for organisms diluted in KCl solutions.

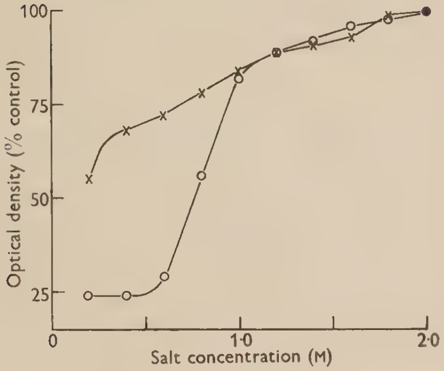

Fig. 5

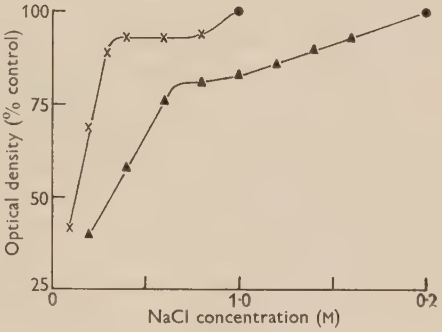

Fig. 6

Fig. 5. Effect of exposing *Vibrio costicolus* before osmotic shock to salt concentrations higher than that in which they were grown. Organisms were grown in media containing M-NaCl and suspended in 2·0 M solutions of NaCl (×) or KCl (○).

Fig. 6. Effect of exposing *Vibrio costicolus* before osmotic shock to NaCl concentration lower than that in which they were grown. ×, grown in 2·0 M-NaCl media and suspended in M-NaCl; ▲, grown in 2·0 M-NaCl and suspended in 2·0 M-NaCl.

It is noteworthy that the organisms had apparently reached osmotic equilibrium on brief transfer to the more concentrated solution, for the upper descending portions of the curves in Fig. 5, continuous with the lower portion, may be taken to indicate reversal of that equilibrium. But the equilibration plainly did not adjust the organisms to the state they would have had if grown at the higher concentration, for the lysis point remained unchanged.

Effect of transfer to lower concentrations of salts. The effect of decreasing the salt concentration of the suspension before the lysis experiment was also investigated. Suspensions of organisms in KCl solutions could not be diluted appreciably without lysis but suspensions in NaCl solutions could be diluted safely to half the concentration of the growth medium. *Vibrio costicolus* grown in media containing 2·0 M-NaCl were suspended in 2·0 M-NaCl. Part of this suspension was used to obtain a control lysis curve. The remainder was diluted with water to give a suspension in M-NaCl and these organisms were immediately subjected to further osmotic shock. Figure 6 shows that this treatment gave a curve identical in shape and lysis point with that obtained with organisms grown in M-salt solution. This type of experiment was repeated with organisms grown in various concentrations of NaCl. In all cases the onset of lysis was related not to the salt concentration during growth but to the subsequent lower concentration in the stock suspension. The period during which organisms remained at the lower salt concentration was very short (less than 2 min.) but, in this time, they apparently reached complete osmotic harmony with the new environment, i.e. a state apparently equivalent to that attained during growth at the lower concentration.

B. *Correlation of lysis with optical density changes*

In the data on the effects of osmotic shock on *Vibrio costicolus* presented so far, it has been assumed that optical density measurements provided an accurate assessment of lysis. Several experiments were performed to test the validity of this assumption.

Reversal of changes in optical density. There was a decrease in optical density of about 10 % as the concentration of NaCl in which the organisms were suspended was reduced from 1·0 to 0·4 M (Fig. 1). If this change were due to osmotic swelling, restoration of the salt concentration to M should restore these optical density values to that of the control. Of organisms which had been subjected to concentrations below the lysis point, only that proportion which had not lysed would be expected to shrink on being transferred to molar solutions. Thus the observed changes in optical density should be reversible with whole organisms and irreversible with lysed preparations. After suspensions had been pipetted into NaCl or KCl solutions in the standard manner, concentrated solutions of the appropriate salt were added to restore the concentrations to molar, the final concentration of organisms was the same in all samples. The new relationship between optical density and the initial degree of osmotic shock is shown in Fig. 7. In NaCl, some reversal of the optical effect occurred at all degrees of osmotic shock. In most suspensions the optical density increased to values above that of the control and there was considerable reversal even in suspensions at concentrations below the lysis point. Since the organisms equilibrate rapidly to lower salt concentrations, the degree of shrinkage when transferred back to a higher concentration would be greater than expected. The highest readings were for organisms which had been subjected to NaCl concentrations between 0·3 and 0·4 M, i.e. at concentrations near the lysis point. Below this point, fewer intact organisms remained, but these could undergo greater shrinkage. Thus the result is a balance between lysis and shrinkage, and the peak of optical density between 0·3 and 0·4 M indicates that this is, in fact, the point at which lysis begins. On the other hand, the optical changes of suspensions diluted in KCl were not reversed under these conditions. It may be concluded that the type of equilibration to lower concentrations found in NaCl suspensions does not occur in KCl suspensions, due perhaps to more permanent damage to the plasma membrane during osmotic shock.

Viability of treated organisms. As a further check on the interpretation of optical density/salt concentration curves, the effect of osmotic shock on viability was investigated. Organisms grown in medium containing M-NaCl were shocked in NaCl solutions and plated on proteose peptone tryptone agar containing M-NaCl. Figure 8 shows that there was a striking loss of viability in NaCl concentrations well above the lysis point. In contrast, there was close agreement between viability and optical density for cells diluted in KCl. The data of Fig. 8 were obtained with very dilute suspensions. The counts were made by direct plating of the experimental suspensions, serial dilutions being unnecessary. When more concentrated suspensions were used, the decrease of viability with decrease in NaCl concentration was much more marked. It appeared, therefore, that organisms transferred to lower salt concentrations were extremely fragile, and that the manipulations involved in serial dilution had a drastic effect on their viability.

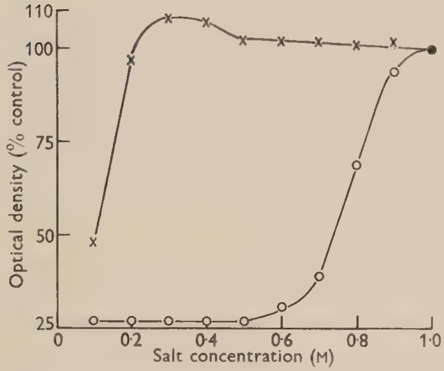

Fig. 7

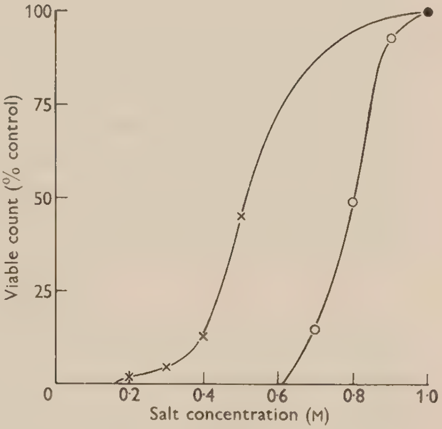

Fig. 8

Fig. 7. Reversibility of osmotic lysis of *Vibrio costiculus*. Organisms from M-NaCl medium were subjected to osmotic shock in NaCl (×) and KCl (○) solutions. The concentrations of shocked suspensions were restored to molar by addition of the appropriate salt and the optical densities measured. The concentrations given in the figure refer to those used to produce the osmotic shock.

Fig. 8. Effect of osmotic shock on the viability of *Vibrio costicolus* suspended in solutions of NaCl (×) and KCl (○). Organisms were grown in medium containing M-NaCl.

The discrepancies between the optical density values and the viability count of shocked organisms cast some doubt on the validity of the former as measurements of degree of lysis. In an attempt to reconcile these differences, organisms subjected to varying degrees of osmotic shock in sodium chloride were examined by phase contrast microscopy.

Microscope examination of osmotically 'shocked' organisms. Organisms from NaCl concentrations between 0·6 and 1·0 M appeared to be normal and intact. Those from concentrations below 0·3 M were mostly disrupted and appeared as ghosts; much debris was also seen in these preparations. Organisms from intermediate salt concentrations were of greatest interest. Some were large, and showed considerable distortion. Close examination revealed that in the majority of such organisms the structural wall had ruptured; the distortion was due to the extrusion of cytoplasm, which appeared to be retained within the plasma membrane, and which bulged through breaks in the cell wall. These breaks usually occurred terminally, but in some were nearly central.

Since the organisms had been harvested in the logarithmic phase of growth, they were in all stages of development, and weak points might be expected to occur at various places in the wall. These structures appear to be identical with the plasmoptysis figures first described by Fischer (1900) and discussed by Knaysi (1951), by Mitchell & Moyle (1956) and Weibull (1956). Fischer observed these bodies in growing cultures of *Vibrio cholerae* and *V. proteus*, but they could also be produced by transferring organisms to hypotonic solutions and by suspending organisms in solutions to whose solutes the plasma membrane was permeable. Fischer deduced that if the cell wall could not swell sufficiently it cracked and the cytoplasm was extruded. Plasmoptysis bodies were not viable.

Thus, in the present experiments, although the cell wall was fractured and the organism no longer viable, the cell membrane continued to hold the cytoplasm. Since lysis consists of the liberation of cytoplasmic contents into the suspending medium, such organisms have not lysed and continue to act as osmometers, as borne out by the reversal of optical density changes on restoration of the original external osmotic pressure. Hence optical density is a reliable guide to the degree of lysis produced by osmotic shock.

DISCUSSION

It will be clear that, though these lytic phenomena are in essence osmotic, they cannot be explained in simply osmotic terms. Lysis was influenced by the cation rather than the anion, and there were outstanding differences in the osmotic sensitivity of the organisms to different cations. Further, the mere removal of water by osmotic means does not, apparently, modify the lysis pattern.

The striking differences in response to NaCl and LiCl on the one hand and to KCl and NH_4Cl on the other find a parallel in data of Pratt & Riley (1955) obtained with a marine bacterium. The superior protection provided by the sodium ion is not attributable to the saline habitat of their strain and ours, since the same difference between cations has been observed with the non-halophilic *Vibrio metchnikovi* (unpublished data). A more likely explanation lies in the greater ease with which the cations of smaller hydrated ionic radii (potassium and ammonium) penetrate the plasma membrane (Boyle & Conway, 1940). The swelling of cells which follows transfer to hypotonic solutions may deform the membrane sufficiently for the smaller particles to enter the cells. The ensuing uptake of water may then result in plasmoptysis. Conversely, the decrease in osmotic sensitivity which followed pretreatment with CTAB was probably the result of leakage of osmotically-active solutes from the cell.

The sharp decrease in degree of viability on dilution is a warning against the use of hypotonic diluents and unsuitable solutes where accurate counts of viable organisms are required. That this dramatic effect is not confined to halophilic bacteria has been demonstrated recently by Marshall & Scott (1958) with *Vibrio metchnikovi*.

Since simple transfer to solutions of higher NaCl concentration did not increase the lysis point of *Vibrio costicolus* there can be no influx of substances tending to bring the internal state of the organisms into adjustment with the new environment, such as must occur during growth. Presumably water is lost on simple transfer but is restored on dilution during the lysis experiment, and hence does not affect the final result. Similar attempts to increase the lysis point of non-proliferating organisms exposed to higher NaCl concentrations by aerobic incubation with oxidizable substrates were unsuccessful even in the presence of potassium phosphates.

The apparently complete adjustment which promptly follows transfer to moderately lower salt concentrations, as shown by the changed lysis point, is probably due to leakage of osmotically active solutes while the organisms are swollen. Such solutes would be small ions or molecules. Bacteria are rich in potassium, and lysis experiments in KCl solutions suggest that this ion penetrates the plasma membrane rapidly when the cell is subjected to an osmotic stress. Hence the change in cellular potassium content was measured when the NaCl concentration of the suspension was halved. About one-third of the potassium was lost, suggesting that this ion may be of major importance in osmotic regulation of bacterial cells. Evidence supporting this suggestion has also been obtained from potassium accumulation studies with *Salmonella oranienburg* (Christian, 1955).

The constant relationship between the NaCl concentrations of the growth medium and of the suspending solution at the onset of lysis shows that the osmotic status of the organism is directly related to that of the environment. Lysis of *Vibrio metchnikovi*, a non-halophile, also begins at NaCl concentrations close to one-third of the concentration during growth (unpublished observations). Hence the internal osmotic status of these two species are similar. This supports the conclusion based on cryoscopic data (Christian & Ingram, 1959) that the explanation of halophilism does not lie in an ability to maintain a relatively small internal concentration of salts.

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REFERENCES

Boyle, P. J. & Conway, E. J. (1940). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1.

Christian, J. H. B. (1955). The water relations of growth and respiration of *Salmonella oranienburg* at 30° C. *Aust. J. Biol. Sci.* **8**, 490.

Christian, J. H. B. & Ingram, M. (1959). The freezing points of bacterial cells in relation to halophilism. *J. gen. Microbiol.* **20**, 27.

Fischer, A. (1900). Die Empfindlichkeit der Bakterienzelle und das baktericide Serum. *Z. Hyg. InfektKr.* **35**, 1.

Knaysi, G. (1951). *Elements of Bacterial Cytology*, 2nd ed. Ithaca, New York: Comstock.

Mager, J., Kuczynski, M., Schatzberg, G. & Avi-Dor, Y. (1956). Turbidity changes in bacterial suspensions in relation to osmotic pressure. *J. gen. Microbiol.* **14**, 69.

Marshall, B. J. & Scott, W. J. (1958). The water relations of *Vibrio metchnikovi* at 30° C. *Aust. J. biol. Sci.* **11**, 171.

Mitchell, P. & Moyle, J. (1956). Osmotic structure and function in bacteria. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 150.

Pratt, D. & Riley, W. (1955). Lysis of a marine bacterium in salt solutions. *Bact. Proc.* **55**, 26.

Robinson, J., Gibbons, N. E. & Thatcher, F. S. (1952). A mechanism of halophilism in *Micrococcus halodenitrificans*. *J. Bact.* **64**, 69.

Robinson, J. & Katznelson, H. (1953). Aspartate-glutamate transaminase in a red halophilic bacterium. *Nature, Lond.* **172**, 672.

Salton, M. R. J. (1951). The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *J. gen. Microbiol.* **5**, 391.

Smithies, W. R., Gibbons, N. E. & Bayley, S. T. (1955). The chemical composition of the cell and cell wall of some halophilic bacteria. *Canad. J. Microbiol.* **1**, 605.

Weibull, C. (1956). Bacterial protoplasts: their formation and characteristics. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 111.

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Eberhart, B. M. & Tatum, E. L. (1959). *J. gen. Microbiol.* **20**, 43–53

A Gene Modifying the Thiamine-requirement of Strains of *Neurospora crassa*

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SUMMARY: A new gene, *thi–1o*, is described in *Neurospora crassa*. This gene is shown to be a modifier of the nutritional response of strains containing the *thi–1* gene, and is not nutritionally detectable in the absence of the *thi–1* mutant locus. The gene *thi–1o* is additionally characterized by a decreased ability to synthesize thiamine from thiazole and pyrimidine; *thi–1o* appears to modify only *thi–1* strains because of their unusually high thiamine requirements. The nutritional responses of strains containing either *thi–2* or *thi–3* loci are not affected by the presence of *thi–1o*. Both *thi–1o* and *thi–4* are anomalous in the condensation of pyrimidine and thiazole. The probability that *thi–1o* may be an isoallele of *thi–4* is based upon a similarity of metabolic pattern and upon location on chromosome III. The failure to obtain recombination in crosses between strains containing *thi–1o* and *thi–4* constitutes additional support for their allelism.

The metabolic pathway of thiamine synthesis in Neurospora is not entirely agreed upon. Tatum & Bell (1946) concluded that thiamine is derived by a condensation of substances equivalent to vitamin pyrimidine (2-methyl-4-amino-5-ethoxymethyl pyrimidine) and vitamin thiazole (4-methyl-5-β-hydroxyethyl thiazole). In subsequent work, Harris (1953) stated that such a scheme does not account for the requirements and the accumulation products of the Neurospora thiamine-requiring mutants. Harris (1955) also proposed an alternative scheme in which a pyrimidine-incomplete-thiazole intermediate is formed prior to the formation of intact thiamine. The quantitative nutritional response of the thiamineless strains is critical in the formulation of either of these alternative proposals. According to Barratt, Newmeyer, Perkins & Garnjobst (1954), the various thiamine-requiring strains of Neurospora have these qualities:

			Growth responses			
Symbol	Isolation no.	Linkage group	Thiamine	Thiazole	Pyrimidine	Pyrimidine & thiazole
thi-1	56501/17084	I	+	−	−	+
thi-2	9185	III	+	−	−	−
thi-3	18558	VII	+	+	−	+
thi-4	85902	III	+	−	−	−

As part of the continued investigation of this problem (Eberhart, 1956), several thiamineless strains have been examined for the presence of modifying genes which might affect quantitative or qualitative requirements of these thiamineless mutants. Although no modifiers were found which contribute to a re-evaluation of the thiamine metabolism in Neurospora, a gene modifying the *thi–1* locus was found in certain wild-type strains. The purpose of this paper is to describe this new gene, *thi–1o*, in terms of its genetic behaviour and metabolic expression.

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METHODS

The basal medium for growing Neurospora was that of Beadle & Tatum (1945). Crosses were made using the chemically defined medium of Westergaard & Mitchell (1947).

Cocarboxylase determinations essentially followed the procedure of Westenbrink & Steyn-Parvé (1950) as follows: 1 g. dried yeast* was suspended in 20 ml. of tris (2-amino-2-hydroxymethyl propane-1:3-diol; Sigma Chemical Co., St Louis, Mo.) buffer at pH 9·0 (0·1 M). The preparation was centrifuged, and three similar washings with distilled water followed quickly. For optimal results this procedure took no more than 30 min. The yeast was then suspended in phosphate buffer (pH 6·2, 0·1 M) with a final volume of 12 ml. One ml. of a 0·1 M-magnesium chloride solution was added, followed by 1 ml. of a thiamine solution (equiv. 100 μg. thiamine). The yeast preparation was stored at 5° until used within 24 hr.). Storage by freezing destroyed only half of the carboxylase activity. One-half ml. of this preparation was added to each Warburg flask (total volume 3 ml.). The substrate consisted of 0·2 ml. of a 2% (w/v) pyruvate solution.

Thiamine and thiamine pyrophosphate were determined by the thiochrome technique of Stiller (1950). Thiochrome was assayed in a Nefluorophotometer (Fisher Scientific Co., New York, N.Y.). In experiments in which the thiamine concentrations were high, direct water-phase thiochrome determinations were successful without the usual isobutanol extraction.

Thiamine synthesis and thiamine phosphorylation were measured by the incorporation of exogenous thiazole and pyrimidine or of thiamine into 'bound' thiamine (i.e. thiochrome not soluble in isobutanol) in mycelia of optimally grown strains of Neurospora. Typically, 5 μg. thiamine/100 ml. were included in the growth media of all strains tested. The strains were grown with agitation for 40 hr. at 30° (exceptions are noted), and filter-sterilized thiazole and pyrimidine or thiamine were then added. After further incubation, the extracts were examined for 'bound' thiamine and for total thiamine (thiochrome soluble after takadiastase treatment).

Air-dried mycelia with carboxylase activity were prepared from Neurospora cultures which were filtered on paper in a Buchner funnel, and washed with three volumes of distilled water. During the final washing the mycelia were suspended so that the resulting pad was spread evenly on the filter-paper. The damp mycelial pad was removed from the funnel and placed on a glass surface. The pad was gently pressed so that it would stick to the glass, and an air stream from an electric fan was used to dry the pad as quickly as possible. When thoroughly dry (coolness of the pad indicates evaporation is still in progress) the mycelial pad was removed from the glass, weighed, and stored in a desiccator in the dark. Mycelial preparations of this type retain carboxylase activity for as long as a year.

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* The authors had no success with dried 'Active' Bakers' yeast preparations, but found air dried Brewers' yeast (Falstaff of San Jose, California) to be satisfactory for cocarboxylase determination.

The carboxylase activity of dry Neurospora mycelia was measured in a Warburg-Barcroft manometer. Weighed samples of mycelium (about 20 mg.) were placed in the manometer flask, and 2·8 ml. of 0·1 M-phosphate buffer (pH 5·6) were added. Pyruvate substrate solution (0·2 ml. of a 2 %, w/v, solution) was added to the side arm. When the closed manometric system reached equilibrium in a 33° water bath, the substrate was added and readings were taken at 5 min. intervals. The first three readings were critical because of the inhibitory effects of the acetaldehyde produced from the pyruvate.

The strains of *Neurospora crassa* used in this study were: (wild types) STA, STa, SY7A, SY4a, (thiamine mutants) 56501a (*thi–1*), 17084–44602 (*thi–1*, pyridoxine), 9185A (*thi–2*), 18558a (*thi–3*), 85902A (*thi–4*). All other strains are derived from crosses of the above strains (Table 1). These strains were obtained from the lyophilized collection at Stanford University, California.

Table 1. *Crosses described in the test*

		Type of auxotrophy*				
Cross no.	Strains in cross	Proto-trophs	Auxo-trophs	B_1 or $Tz+Py$	B_1	B_1 or Tz
56501–3	56501a × SY7A (*thi–1 × thi–1o*)	59	59	37	22	—
56501–6	56501–3–69A × 9185a (*thi–1; thi–1o × thi–2*)	47	46	46	5	41
56501–7	56501–5(11–5) × SY7A (*thi–1; thi–3 × thi–1o*)	(See text)				
56501–17	56501–3–90A × 56501–3–88a (*thi-1 × thi–1; thi–1o*)	—	—	—	—	—
18558–1	18558A × 56501–7–54a (*thi–3 × thi–1o*)	35	34	—	—	34
85902–2	85902A × 56501–3–88a (*thi–4 × thi–1; thi–1o*)	15	55	—	55†	—
85902–3	85902A × 56501–3–93a (*thi–4 × thi–1*)	26	64	28	36	—
9185–1	STA × 9185a (*wild × thi–2*)	0	69	—	69	—

* Will grow on: B_1 = thiamine, Tz = thiazole, Py = pyrimidine.

† 15 *thi–1, thi–1o* and 40 *thi–4* strains as determined by 5 day growth response.

RESULTS

Since the possibility of the existence of modifier genes in *thi–1* stocks had never been adequately investigated, a cross of *thi–1* and wild type SY7A was made (cross 56501–3). In the random isolates of this cross a number of novel thiamine-requiring strains were found, which were characterized by their inability to grow on thiazole and pyrimidine (either together or separately) in a standard 3–day assay at 25°. The requirements of these new strains were therefore qualitatively similar to those of *thi–2* or *thi–4* strains, while their quantitative responses to intact thiamine remained quite characteristic of normal *thi-1* strains (Table 2). Subsequent investigation showed that a new gene, *thi-1o*, is responsible for this modification of the *thi–1* phenotype in these unusual strains. This gene apparently came from SY7A stocks.

Table 2. *The growth response of isolates from cross* 56501–3

Test cultures grown three days at 25° in 20 ml. minimal with supplements noted above.

	Isolate number				
Additions (μg./20 ml.)	8 (*thi–1*)	40 (*thi–1*)	69 (*thi–1; thi–1o*)	71 (*thi–1; thi–1o*)	77 (*thi–1*)
	dry wt. mycelium (mg.)				
Thiamine					
0	6	4	4	2	5
0·05	14	12	14	13	14
0·1	16	15	16	18	20
1·0	40	43	49	45	42
5·0	63	66	70	56	75
20·0	65	72	80	64	70
Thiazole					
0·05	4	3	2	4	5
0·1	4	5	3	3	4
1·0	6	5	3	4	6
5·0	7	7	3	3	8
20·0	13	13	5	5	15
Pyrimidine					
5·0	4	4	3	3	4
20·0	4	3	3	2	2
Thiazole + pyrimidine					
0·1 0·05	6	+0	3	2	6
0·5 0·05	8	7	3	2	6
1·0 0·05	9	8	3	2	6
10·0 0·05	12	12	4	6	14

Mechanism of thi–1o action

Additional insight into the metabolic basis of the *thi–1o* modifier was sought by studying the thiamine synthesizing abilities of various strains containing the *thi–1o* modifier. The steps involved in the biological condensation of thiazole with pyrimidine, and the subsequent phosphorylation of thiamine, were investigated directly. In designing a test system for the *in vivo* thiamine metabolism of these strains, there were certain general considerations involved. The *in vivo* thiamine metabolism has at least three steps: the condensation of thiazole with pyrimidine, the phosphorylation of thiamine, and the binding of thiamine diphosphate within the cell. Since it is not yet possible to block any of these steps successfully without interfering with metabolism in general, an examination of the over-all system is required.

For example, the thiazole-pyrimidine condensing system must be measured in terms of 'bound' thiamine, because the ability of Neurospora strains to phosphorylate and maintain thiamine becomes a potentially limiting factor when bound thiamine is used as an index of pyrimidine and thiazole condensation. The fate of exogenous thiamine was, therefore, considered to be the first logical step in the study of these systems. Figure 1 illustrates the ability of several Neurospora strains to take up and maintain thiamine. Each culture was grown for 40 hr. at 30° on minimal medium + 0·05 µg. thiamine/ml. and then incubated with 50 µg. thiamine. The uptake and maintenance of thiamine by these strains are quite similar despite their extremely different thiamine requirements.

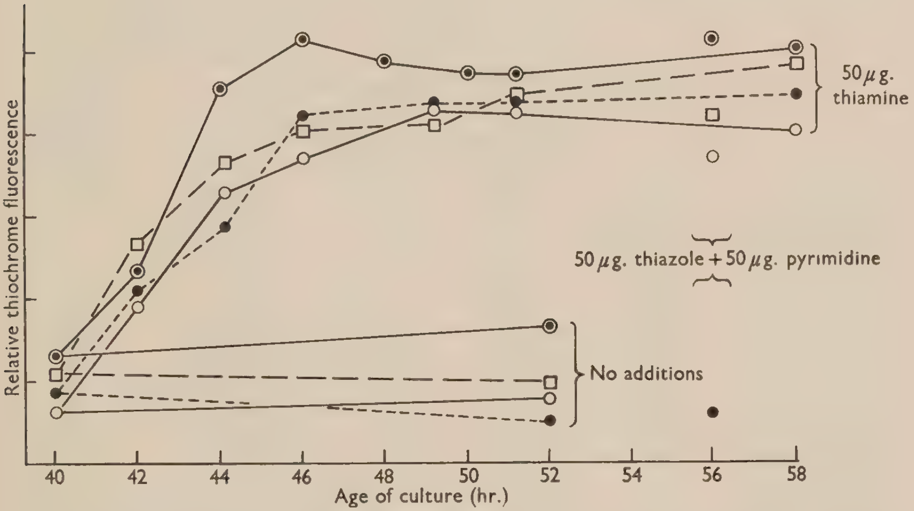

Fig. 1. Thiamine synthesis by Neurospora. Cultures were grown 40 hr. at 30° with 2·5 µg. thiamine/50 ml. minimal medium. At 40 hr. 50 µg. thiamine (*a*) or 50 µg. thiazole + 50 µg. pyrimidine (*b*) were added. In control flasks there were no additions (*c*). Strains used were: □, *thi–1* (56501a); ●, *thi–1*; *thi–1o* (17084–1–3); ○, *thi–3* (18558A); ⊙, *thi–1o* + (STA).

A test for the presence of the *thi–1* gene in Neurospora strains was devised by incubating optimally-grown mycelia cultures in the presence of 50 µg., each of thiazole and pyrimidine and measuring the thiamine synthesized by thiochrome fluorescence. The results are also shown in Fig. 1. With the exception of the *thio–1o* containing strain, these cultures produced similar amounts of thiamine from exogenous pyrimidine and thiazole. The *thi–1o* containing strain was unable to produce a significant amount of thiamine from its precursors. These results and the results in Table 3 indicate that this test is a valid means of detecting the presence of *thi–1o* in strains independently of nutritional tests.

Other strains were tested for their ability to condense pyrimidine and thiazole. The experiment illustrated in Fig. 2 shows the relative synthetic rates of a few of these strains. The substrates of 100 µg. each of pyrimidine and thiazole were introduced into 48 hr.-old mycelial cultures grown in the standard optimal media. Both *thi–1* and *thi–3* strains show good rates of thiamine synthesis, while the *thi–2* and *thi–1o* strains show very little increase in thiamine. Interestingly, even the SY7A (*thi–1o*) strain had relatively low activity in this test system, despite the fact that the growth of this wild-type strain was unaffected by thiamine.

Table 3. *The pyrimidine and thiazole condensing abilities of Neurospora mycelia*

Cultures were grown in 300 ml. Erlenmeyer flasks with 150 ml. minimal medium + 5 µg. thiamine/100 ml. Cultures were shaken at 30° for 41 hr., then 100 µg. of pyrimidine and thiazole were added aseptically and the cultures were shaken for another 12 hr.

Strain		No addition		Pyrimidine 100 µg., thiazole 100 µg.	
		Final dry wt. mycelium (mg.)	Relative thiochrome fluorescence	Final dry wt. mycelium (mg.)	Relative thiochrome fluorescence*
56501a	*thi–1*	0·386	8	0·372	35
56501–3–70a	*thi–1*	0·449	9	0·478	50
56501–3–71A	*thi–1*; *thi–1o*	0·350	6	0·400	8
56501–3–75a	*thi–1*	0·384	6	0·360	34
56501–3–77a	*thi–1*	0·378	9	0·455	45
56501–3–86A	*thi–1*	0·370	9	0·400	33
56501–3–87A	*thi–1*	0·380	7	0·413	32
56501–3–88a	*thi–1*; *thi–1o*	0·450	8	0·420	9
56501–5–(15–8)	*thi–3*	0·434	4	0·440	38
56501–5–(15–5)	*thi–1*	0·430	8	0·480	40
56501–5–(15–3)	*thi–1*	0·448	8	0·460	40

* Relative fluorescence here is directly proportional to thiamine (thiochrome) concentration in the mycelium.

Since the nutritional response of a 'wild-type' strain was unmodified by the presence of *thi–1o*, it was of interest to study the effect of *thi–1o* on the *thi–3* nutritional phenotype. This was done by making crosses which would result in *thi–3*; *thi–1o* double mutants and testing the nutritional and thiamine synthesizing capacities of various random isolates. Results of two experiments are reported.

In the first experiment a *thi–1*; *thi–3*; *thi–1o*+ double mutant was crossed to *thi–1o* (SY7A), (cross 56501–7). Strains were randomly isolated. The following nutritional types of strains were found: 'wild', *thi–1*, *thi–3*, and strains which responded like *thi–1*; *thi–1o*. In thiamine-synthesizing tests, both the 'wild' and *thi–3* types showed either high or low activity (+ or − *thi–1o*), while the strains which were like *thi–1* nutritionally showed only high thiamine synthesizing abilities. All strains which responded nutritionally like *thi–1*; *thi–1o* doubles had low thiamine-synthesizing capacities. Typical thiamine-synthesizing abilities of some of these strains are shown in Table 4. These results are consistent with the idea that *thi–1o* drastically lowers the ability of *thi–3* strains to condense pyrimidine and thiazole, without modifying their nutritional requirements. A similar decrease in synthetic capacity by *thi–1o* in *thi–1* strains results in a change in nutritional requirements.

A second experiment was designed to show the interactions of *thi–1o* and *thi–3* genes. Strain 18558A (*thi–3*), which did not contain *thi–1o*, was crossed (cross 18558–1) to a thiamine-independent strain (56501–7–54a) which contained *thi–1o* as shown by a lowered ability to condense pyrimidine and thiazole. Of the seventy random spores dissected from this cross, thirty-six were wild and thirty-four were like *thi–3* nutritionally. Several isolates were examined for their synthetic abilities and bound thiamine (thiamine pyrophosphate) was measured in terms of the relatively specific yeast cocarboxylase assay. The data given in Table 5 show the cocarboxylase-synthesizing abilities of *thi–1o*+, *thi–3*; *thi–1o* and *thi–3* strains. In the longer growth and incubation of these cultures, *thi–1o* can be shown not to be a complete block. Nevertheless, under these conditions there is still at least a twofold difference in the synthetic performance of modified and unmodified strains. The *thi–3*; *thi–1o* strains are nutritionally similar to *thi–3* in their ability to respond to exogenous thiazole as a source of thiamine.

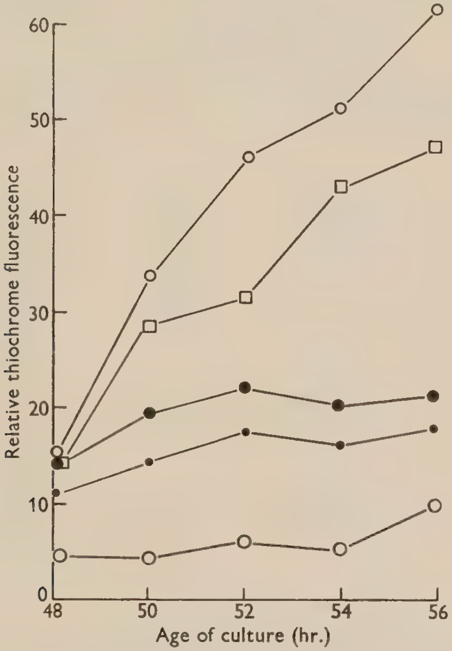

Fig. 2. Thiamine synthesis from pyrimidine and thiazole by Neurospora. Cultures were grown on 50 ml. minimal medium +2·5 μg. thiamine. At 48 hr. 100 μg. thiazole+100 μg. pyrimidine were added. Strains used were: □, *thi–1* (56501a); ●, *thi–1*; *thi–1o* (17084–1–3); ○, *thi–2* (9185a); o, *thi–3* (18558A); •, *thi–1o* (Sy7A).

It was thought that carboxylase activities in these strains might be a useful index of the presence of *thi–1o* in its role as modifier of cocarboxylase synthesis from thiazole and pyrimidine. Yet, this test system was not applicable to optimally grown mycelia since maximal carboxylase activity is found in *thio–1o* strains. Table 5 includes the carboxylase activities of *thi–1o+*, *thi–3*; *thi–1o* and *thi–3*. These results indicate that *thi–1o* is not distinguishable in terms of the carboxylase activities of optimally grown strains. Carboxylase activity apparently does not reflect differences of internal cocarboxylase concentrations at these high cocarboxylase levels. The apocarboxylase in these mycelia is probably saturated with coenzyme at the high cocarboxylase

Table 4. *Thiamine-synthesizing abilities* of Neurospora isolates from cross* 56501–7

Cultures were grown in shake culture in 50 ml. of minimal medium +2·5 μg. thiamine. At 40 hr. cultures were supplemented with 50 μg. each of pyrimidine and thiazole, shaken an additional 12 hr., then harvested.

		Additions at 40 hr.		
		None	Thiamine	Thiazole + pyrimidine
Strain	Nutritional phenotype†	Relative thiochrome concentrations*		
56501–7–20	*thi–1*	5	24	48
23	*thi–1*	9	58	48
34	*thi–3*	5	49	51
64	*thi–3; thi1o*	4	39	8
72	*thi–1; thi–1o*	3	55	12
75	*thi–1; thi–1o*	4	60	13
76	*thi–1; thi–1o*	6	51	11
77	*thi–3; thi–1o*	7	44	10
56501–3–69	*thi–1; thi–1o*	8	39	14
71	*thi–1; thi–1o*	5	42	12
73	*thi–1; thi–1o*	7	60	18
87	*thi–1*	4	26	35
SY7A	*thi–1o*	10	54	13
9185a	*thi–2*	2	30	4

*Values in table are in relative thiochrome concentrations (see methods).

† *thi-3*; *thi–1o* doubles are nutritionally identical to *thi–3* strains; *thi–1*; *thi–1o* strains are quantitatively like *thi–1* (high thiamine requirement), but they will eventually show growth on thiazole- and pyrimidine-containing media at times when the minimal cultures are still blank.

Table 5. *Cocarboxylase synthesis and carboxylase activity in modified and unmodified Neurospora strains*

Cultures were grown with shaking in 50 ml. of minimal medium + 2·5 μg. thiamine in 125 ml. Erlenmeyer flasks at 30°. After 46 hr. of growth, solutions containing 50 μg. thiamine, or 50 μg. each of pyrimidine and thiazole, sterilized by filtration were added and the cultures were shaken an additional 14 hr. and harvested.

Values in the table are: $a = \mu g.$ cocarboxylase/g. dry wt. mycelium; $b =$ carboxylase activity as μl. CO_2/25 mg. dry wt. mycelium/5 min. (average of three readings).

		Additions at 46 hr.					
		None		Thiazole 50 μg. + pyrimidine 50 μg.		Thiamine 50 μg.	
Strain	Genetic composition	*a*	*b*	*a*	*b*	*a*	*b*
STA	*thi–1o+*	37	158	105	172	105	168
18558–1–9	*thi–3; thi–1o+*	8	78	86	140	92	143
18558–1–8	*thi–3; thi–1o*	16	105	54	147	96	150
18558–1–10	*thi–3; thi–1o*	19	110	41	110	101	112

concentrations resulting from optimal growth. From these experiments and others it was seen that the presence of *thi–1o* suppresses the condensation of pyrimidine and thiazole in every strain tested. The nutrition of *thi–1* strains is modified by *thi–1o*, while the nutritional responses of wild-type (Table 6) and *thi–3* strains are not impaired by *thi–1o*. Consequently the only means of detecting *thi–1o* in *thi–3* and wild-type stocks is a test of the synthetic abilities of their mycelia.

Table 6. *Growth response of Neurospora strains thi–1o and thi–1o+ to thiamine-active substances*

The strains were grown 45 hr. at 30° in 125 ml. Erlenmeyer flasks containing 20 ml. minimal medium + supplements indicated. Figures represent dry weight of mycelium in mg.

		Supplements added (2 μg./20 ml.)			
		None	Thiamine	Thiazole	Pyrimidine
Strain Number	Genotype	Dry wt. mycelium (mg.)			
56501–7–54a	*thi–1o*	34, 34	33, 34	32, 31	31, 29
56501–7–40A	*thi–1o*	19, 16	17, 17	18, 15	14, 13
SY7A	*thi–1o*	39, 38	38, 42	41, 38	38, 38
STa	*thi–1o+*	57, 48	51, 51	50, 46	46, 51
STA	*thi–1o+*	56, 53	54, 55	55, 60	60, 49

Genetic relationships of thi–1o

Since *thi–1o* expresses itself as a nutritional anomaly only in the presence of *thi–1*, a cross was made (cross 56501–17) between two *thi–1* strains, one of which contained *thi–1o*. In fourteen complete asci tested, *thi–1o* showed only first division segregation, which indicated a map location close to the centromere. The 2:2 segregation data found indicated that the *thi–1o* character was associated with a single gene. Additionally, the data from the original cross (cross 56501–3) in which *thi–1o* segregated from SY7A are also consistent with the hypothesis that *thio–1o* was a single gene in this 'wild-type' stock.

When *thi–1o* was detected in *thi–1* stocks, it became important to know whether it was present in the various other thiamine-requiring stocks. The presence of *thi–1o* in *thi–2* or *thi–4* stocks would make obtaining and studying of double mutants with *thi–1* extremely difficult.

A cross of a *thi–1*; *thi–1o* strain by a *thi–2* strain (cross 56501–6) was made, and a number of recombinant *thi–1* strains were recovered which did not contain *thi–1o*, thus showing that *thi–1o* is not present in the *thi–2* parent (9185*a*), and that *thi–2* is not allelic with *thi-–1o*. In a cross of *thi–4* by *thi–1* (without *thi–1o*) (cross 85902–3), the randomly selected unmodified *thi–1* types were approximately equal to the wild types suggesting there are no modifiers in *thi–4* not linked to *thi–4*.

The possibility that *thi–1o* might be linked to or allelic with *thi–4* was considered because of the similarity of action of these loci. From the linkage data summarized by Barratt *et al.* (1954), *thi–1* is in linkage group I, *thi–2* and *thi–4* are in group III, while *thi–3* is in group VII. A cross of a *thi–4* strain by *thi–1*; *thi–1o* strain (cross 85902–2) was made, and of fifty-five thiamine-requiring random isolates, not one was a recombinant *thi–1*; *thi–1o+* nutritional type. Of these fifty-five isolates forty were *thi–4*-containing strains, and fifteen were *thi–1*; *thi–1o* parental types. The parental *thi–1*; *thi–1o* types could be differentiated from isolates which contained *thi–4* because the *thi–1*; *thi–1o* eventually grew on test media containing pyrimidine and thiazole in 5–6 days at 30°. Strains containing the *thi–4* gene did not grow under comparable conditions. These results were in agreement with the proposed parental genotypes. These results are also consistent with the location of *thi–1o* in linkage group III. Since neither *thi–4* or *thi–1o* have shown recombination with their centromere nor with each other, and since their function is at the same metabolic step, their allelism is strongly suggested.

DISCUSSION

Considerable attention has been paid to the mechanism of gene suppressor action in Neurospora since the initial work of Houlahan & Mitchell (1947). This is mainly due to the relative ease of obtaining this type of modifier. Relatively little attention has been directed to other types of modifiers in Neurospora. There is no reason at present to believe that the basis of suppressor action is fundamentally different from that of other modifiers. The difference may only be a definitional one concerning the direction (towards or away from wild-type phenotype) of the modification rather than a fundamental difference in mechanism.

Modifier action can be considered as operating at two levels: (*a*) at the enzyme-forming level where apoenzyme specificity is determined; (*b*) at the enzyme activity level, where co-factor, pH, substrate and other factors are involved. While some of these inter-relationships have been shown in suppressor systems (Yanofsky 1956) the examples from other modifier systems are limited. Haskins & Mitchell (1952) and Newmeyer & Tatum (1953) studied modifiers in Neurospora which apparently have their effect at the substrate level. These investigators independently found that the phenotype of a gene (*nt*), which is involved in nicotinic acid and tryptophan biosynthesis, is affected both qualitatively and quantitatively by the presence of modifying genes. Strauss (1956) studied the gene *at* which acts as a modifier of succinate (*suc*) requiring strains. Strains containing *at*; *suc* are unable to grow immediately on acetate, while *suc* strains can use this substrate.

The gene *thi–1o* appears to illustrate gene modifier action at the substrate level. The *thi–1o* mechanism of modification in the strains where it has been studied is clearly that of modulating the ability to condense pyrimidine with thiazole. Still *thi–1o* is a modifier in the strictest sense, because its primary phenotypic expression is not discernible in terms of the wild-type nutritional phenotype, while it specifically modifies the nutritional phenotype of the *thi–1* strains tested. The fact that thiamine mutants derived from only 17084 and 56501 strains are modified by *thi–1o* is in agreement with their probable allelism. The *thi–1o*; *thi–1* mutant strains are similar to *at*; *suc* strains in that they eventually grow on thiazole + pyrimidine just as *at*; *suc* strains will eventually grow on acetate. As with *thi–1o* strains, the *at* strains show a wild-type growth rate.

The gene *thi–1o* is also of interest as a probable isoallele of *thi–4*. In this role it represents a class of subthreshold alleles in a series which would range from mutants possessing a complete block through 'leaky-mutants' to mutants with no auxotrophic requirements at all. Members of this last class are most poorly represented in the catalogue of mutant types because simple nutritional techniques are inadequate to detect them. Subthreshold alleles (or wild-type isoalleles) can be picked up by specific physiological techniques as with the sub-threshold tryptophan mutants studied by Giles (1955). Alternatively, as suggested by the work of Goldschmidt & Piternich (1956) with the effects of subthreshold alleles on phenocopies in *Drosophila*, and as indicated by the work with *thi–1o*, these isoalleles may be detected by their interaction with other genes like *thi–1* which can lower the nutritional threshold to a point where the subthreshold gene can express itself.

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REFERENCES

BARRATT, R. W., NEWMEYER, D., PERKINS, D. D. & GARNJOBST, L. (1954). Map construction in *Neurospora crassa*. *Advanc. Genet.* **5**, 1.

BEADLE, G. W. & TATUM, E. L. (1945). *Neurospora* II. Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* **32**, 678.

EBERHART, B. M. (1956). *The Biochemical Genetics of Thiamine Metabolism in* Neurospora crassa. Doctoral thesis, Stanford University.

GILES, N. H. (1955). Forward and back mutation in specific loci in *Neurospora*. *Brookhaven Symp. Biol.* **8**, 103.

GOLDSCHMIDT, R. B. & PITERNICK, L. K. (1956). New experiments on chemical phenocopies. *Proc. nat. Acad. Sci., Wash.* **42**, 299.

HARRIS, D. L. (1953). Biosynthesis of thiamine in *Neurospora*. *Fed. Proc.* **12**, 214.

HARRIS, D. L. (1955). Alternative pathways in thiamine biosynthesis in *Neurospora*. *Arch. Biochem. Biophys.* **57**, 240.

HASKINS, F. A. & MITCHELL, H. K. (1952). An example of modifying genes in *Neurospora*. *Amer. Nat.* **86**, 231.

HOULAHAN, M. B. & MITCHELL, H. K. (1947). A suppressor in *Neurospora* and its use as evidence for allelism. *Proc. nat. Acad. Sci., Wash.* **33**, 223.

NEWMEYER, D. & TATUM, E. L. (1953). Gene expression in *Neurospora* mutants requiring nicotinic acid or tryptophan. *Amer. J. bot.* **40**, 392.

STILLER, E. T. (1950). In *Vitamin Methods*, I, p. 95. Ed. P. Gyorgy, New York: Academic Press, Inc.

STRAUSS, B. S. (1956). The nature of the lesion in the succinate requiring mutants of *Neurospora crossa*: interaction between carboxylase and nitrogen metabolism. *J. gen. Microbiol.* **14**, 494.

TATUM, E. L. & BELL, T. T. (1946). *Neurospora*. III. Biosynthesis of thiamin. *Amer. J. Bot.* **33**, 15.

WESTENBRINK, H. G. K. & STEYN-PARVÉ, E. P. (1950). Methods for the determination of diphosphothiamine. *Z. Vitaminforsch.* **21**, 461.

WESTERGAARD, M. & MITCHELL, J. K. (1947). *Neurospora*. V. A synthetic medium favouring sexual reproduction. *Amer. J. Bot.* **34**, 573.

YANOFSKY, C. (1956). Gene interactions in enzyme synthesis. In *Enzymes, Units of Biological Structures and Function*, p. 147. Ed. Gaebler, O. H. New York: Academic Press.

Olitzki, A. L. & Godinger, D. (1959). *J. gen. Microbiol.* **20**, 54–60

Investigation on Streptomycin Inhibitors with the Aid of a Streptomycin-dependent *Vibrio comma*

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SUMMARY: The growth and amylase activity of a streptomycin-dependent *Vibrio comma* on starch agar plates was gradually decreased with decrease of the streptomycin-concentration. The concentrations of streptomycin and the streptomycin-antagonizing properties of bacterial products also could be assayed by a cup plate method. The diameters of the zones of decomposed starch around the cups were proportional to the logarithms of streptomycin concentration. In the presence of a constant concentration of streptomycin they were inversely proportional to the concentration of bacterial antagonist. The following species and genera of micro-organisms produced antagonists (in decreasing order of their activity): *Pseudomonas* sp., *Proteus morganii, Clostridium botulinum, C. parabotulinum, C. welchii, C. septicum, Bacillus megaterium, B. subtilis, Rhodotorula* sp., and some strains of the genera *Escherichia, Shigella* and *Klebsiella*. No inhibitory effects were observed with products of *Salmonella paratyphi* A and B, *Serratia marcescens, Staphylococcus aureus, S. albus, Streptococcus faecalis, S. lactis, Brucella abortus, B. melitensis, Pasteurella pestis, P. pseudotuberculosis, Alkaligenes faecalis, Saccharomyces* sp., *Candida albicans*.

Recent investigations by Olitzki & Olitzki (1957) revealed that at low concentrations of streptomycin the amylase activity of a streptomycin-dependent strain of *Vibrio comma* was not developed. This inhibition was, therefore, used by them as an indicator in assaying streptomycin in liquid media. In the experiments reported this method was simplified by introducing a cup plate method which permitted the quantitative determination of concentrations of streptomycin and the antagonistic activities of different preparations against a fixed standard concentration of streptomycin.

The first investigations on antagonists of streptomycin were carried out by Lightbown (1950) who described a rapid method of demonstrating streptomycin antagonist activity in bacterial cultures. As indicator organisms *Bacillus subtilis* or *Staphylococcus aureus* were used. By incorporating both antibiotic and indicator organisms in nutrient agar, the antagonistic power of culture filtrates was assayed by a cup-plate method. The diameter of the zone of growth around the cup was proportional to the logarithm of the concentration of the antagonist, which was formed during the growth of *Pseudomonas aeruginosa* in chemically-defined medium and in ordinary culture medium. Lightbown (1954) isolated material produced by *Pseudomonas aeruginosa* which antagonized the activity of streptomycin, and which itself inhibited bacterial growth in low concentrations. The antagonist was produced by most strains of *P. aeruginosa* under favourable conditions. Strains of *Staphylococcus aureus, Escherichia coli, Serratia marcescens, Bacterium bodenheimer,* streptococci of Lancefield's group D, *Bacillus subtilis* and *B. pumilus* did not produce similar activity. The purpose of the work reported below was to determine whether the production of streptomycin antagonists is limited to the genus *Pseudomonas* or shared by other micro-organisms.

METHODS

Strains employed for production of inhibitors. Forty-five bacterial strains were investigated for their ability to produce antagonists to streptomycin. The vibrios used were described in detail elsewhere (Olitzki & Olitzki, 1955, 1957) and by Sulitzeanu & Olitzki (1955). The indicator organism was the streptomycin-dependent strain of *Vibrio comma*, strain Ogawa 40.

Growth of the organisms. The aerobic and facultative anaerobic strains were grown on the surface of nutrient agar in Roux bottles from an inoculum of 2×10^9 bacteria suspended in 5 ml. of saline.

The anaerobes were cultivated on a medium which contained (%, w/v): 0·05, sodium mercaptoacetate; 0·025, cysteine; 2·5, enzymically-hydrolysed caseine; 0·05, tryptophan; 0·5, glucose; 0·0000005, biotin; 0·0000005, thiamine; 0·00001, *p*-aminobenzoic acid; (%, v/v) 1·0, solution A; 0·1, solution B. Solution A contained (%, w/v): 10·0, K_2HPO_4; 10·0, KH_2PO_4. Solution B contained (%, w/v): 4·0, $MgSO_4.7H_2O$; 0·2, NaCl; 0·2, $FeSO_4.7H_2O$; 0·2, $MnSO_4.4H_2O$.

Production of the inhibitory substances. After 48 hr. of incubation at 37° the fluid which was still present in the bottles of the aerobic-growing organisms was removed and filtered through a Seitz pad. The bacteria which remained on the surface were suspended in a minimal volume of buffered saline solution (pH 7·4) which just permitted a complete removal of the whole bacterial growth. The bacterial suspensions were exposed for 30 min. to the action of a Raytheon Sonic Vibrator (9 kcyc.), the debris centrifuged down and the supernatant fluids tested for their protein content. All extracts were brought to a dilution which contained 10 mg. protein/ml.

The anaerobic bacteria were removed by centrifugation from their medium after 24 hr. of incubation at 37°, resuspended in a small volume of the same medium to give a suspension of 10^9 bacteria/ml., incubated for 48 hr. at 37°, and the supernatant fluids examined for their inhibitory action.

Contact of streptomycin with inhibitors. One-tenth ml. of the final products (the supernatant fluids and the cell extracts) were incubated for 3 hr. with 0·9 ml. of a streptomycin solution (final streptomycin concentration of 20 μg./ml.).

Assay of activity of the resulting streptomycin solution. After a 3 hr. exposure at 37° 0·2 ml. portions of the solution were transferred to glass cups placed on the surface of a nutrient agar plate. The nutrient agar contained (%, w/v): Bacto peptone, 1·0; NaCl, 0·5; Na_2HPO_4, 0·25; Bovril, 0·3; starch 0·5; agar, 2·5; (the pH was adjusted to 8·5).

This medium was poured into plates of different sizes with a bacterial suspension to give 5×10^8 vibrios/ml. agar. After incubation for 24 hr. an iodine solution was poured on the surface of the plates and the diameter of the white zone resulting from the decomposition of starch was measured. A concentration of 20 μg. streptomycin/ml. produced an average diameter of 20·8 mm. and by comparison with this standard the antagonist activity of the different preparations was determined (see Plate 1).

The reliability of the method used. Figure 1 shows the relationship between log concentration streptomycin in a range from 2·5 to 20·0 μg./ml. and the diameters of the zones of decomposed starch which appeared as white zones by treatment of the plates with iodine. The average diameters, for 5, 10 and 20 μg. streptomycin/ml., determined from 28 repeated examinations, were 14·8, 18·3, and 20·8 mm., respectively, the respective standard deviations were ±0·9, ±1·13 and ±1·44 mm. Under these circumstances a decrease of the zone diameter from 20 to 18 mm. should indicate a 50 % and a decrease to about 15 mm. a 75 % loss in activity of streptomycin. On the other hand, there appeared in the presence of 20 μg. streptomycin/ml. and dilutions 1/32, 1/16, 1/8, 1/4, and 1/2 of an inhibitory preparation of *Pseudomonas aeruginosa* the following respective diameters of white zones (mm.): 20·8, 18·1, 14·7, 8·8, 0. According to Fig. 1 the respective concentrations of active streptomycin were exactly 20, 10, 5, 2·5 and 0 μg./ml. Thus this experiment proved that in a certain range of dilutions the diameters of the zones of starch decomposition were inversely proportional to the logarithm of the concentration of antagonist.

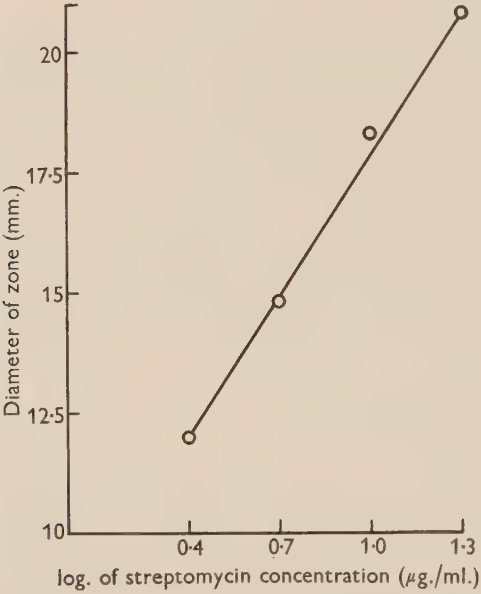


Fig. 1. The diameters of the zones of starch decomposition (mm.) in relation to the logarithms of the streptomycin concentrations.

RESULTS

Preparations of antagonists of different origins

Table 1 shows the effects of several antagonistic bacterial products revealed by this method. The most active strains were found in the genus *Pseudomonas*. Four out of 9 strains effected a complete inactivation, 4 others an inactivation of 75 %, and one strain of 50 %. The supernatant fluids resulting from centrifugation of culture fluids showed almost the same degrees of activities as the cell-free extracts obtained after disruption of the organisms by sonic waves. However, the antagonistic actions were not limited to the genus *Pseudomonas*. Two out of 5 vibrios (Inaba and Celebes) showed some

Table 1. *Effect of bacterial products on zone of starch decomposition produced by* Vibrio comma (*Ogawa* 40, *streptomycin-dependent*) *in the presence of a standard concentration of* 20 *μg. streptomycin/ml.*

Strain tested	Diameter of zone of decomposition (mm.) in the presence of: Supernatant fluids	Cell-free extracts
Pseudomonas hydrophila	13·0	14·0
P. non-liquefaciens	14·0	15·0
P. fluorescens	15·0	12·0
Pseudomonas sp. from eggs	15·0	15·0
P. aeruginosa	0	0
Pseudomonas sp.	17·0	19·0
Pseudomonas sp. 46/4	0	0
Pseudomonas sp. 56/1	0	0
Pseudomonas sp. 84/2	0	0
Vibrio comma (Inaba)	18·0	20·0
V. comma (Celebes)	18·5	20·0
V. comma (Ogawa 1)	19·5	20·0
V. comma (Ogawa 40)	19·0	20·0
Vibrio sp.	21·0	19·0
Proteus vulgaris V_1	18·0	17·5
P. vulgaris V_2	18·5	18·0
P. morganii M_1	15·0	16·5
P. morganii M_2	19·5	18·5
P. rettgeri R_1	20·0	18·0
P. rettgeri R_2	18·0	18·5
Escherichia coli	18·5	15·0
Klebsiella sp.	19·0	17·0
Shigella flexneri	20·0	16·5
Clostridium parabotulinum 3732	—	14·4
C. welchii 8798	—	13·5
C. botulinum	—	13·5
C. septicum	—	14·5
C. histolyticum 503	—	18·0
Bacillus megaterium	21·0	15·0
B. subtilis	20·0	17·0
Rhodotorula sp.	15·5	20·0

Diameter of the zone of decomposition in the presence of streptomycin 20 μg./ml. without inhibitor was 20·8 mm.

antagonistic effects, decreasing the activity of streptomycin to about 50 % of it original value. Another group of micro-organisms able to produce inhibitor was found in the genus *Proteus*. The cell-free extracts of 6 strains decreased the activity of streptomycin to about 50 % and one strain, *Proteus morganii* even to about 75 %.

The extracts obtained by autolysing of 4 clostridia decreased the activity of streptomycin to about 75 % and of *Clostridium histolyticum* to 50 % of its original value. The supernatant fluids of 3 strains of Enterobacteriaceae (*Escherichia coli*, *Klebsiella* sp., *Shigella flexneri*) exerted a relatively weak effect. However, in the presence of their sonic extracts nearly 75 % of streptomycin was inactivated. The supernatant fluids of two cultures of the genus *Bacillus* were inactive. However, the cell-free extracts of the same strains decreased the activity of streptomycin by more than 50 %. On the other hand, the supernatant fluid of a *Rhodotorula* sp. showed some antagonistic activity, while its sonic extract was inactive. Table 2 shows results obtained with the products of other micro-organisms which were almost inactive in this test.

Table 2. *Starch decomposition by the streptomycin-dependent strain Ogawa 40 in the presence of different inactive or slightly active bacterial products at a standard concentration of 20 μg. streptomycin/ml.*

	Diameter of zone of decomposition (mm.) in the presence of	
Strain tested	Supernatant fluids	Cell-free extracts
Salmonella paratyphi A	19·0	21·0
S. paratyphi B	21·5	20·0
Serratia marcescens	18·5	22·0
Staphylococcus albus	20·0	18·0
S. aureus	21·0	18·0
Streptococcus faecalis	17·5	20·0
S. lactis	20·5	20·0
Brucella abortus	18·5	18·5
B. melitensis	18·5	19·5
Pasteurella pestis	20·0	18·5
P. pseudotuberculosis	18·0	19·0
Alkaligenes faecalis	18·0	18·0
Saccharomyces sp.	18·0	20·0
Candida albicans	18·0	20·0

Control of the bacterial products for growth-inhibitory activity

In order to avoid false interpretation of the results caused by growth-inhibitory bacterial products, we tested the antagonistic products for their antibiotic activity in the absence of streptomycin against the following bacteria: *Vibrio comma* (Ogawa 1, streptomycin-sensitive) which was the parent strain of the dependent strain Ogawa 40, *Bacillus subtilis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Neisseria catarrhalis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella typhi*, and *Streptococcus pyogenes*. In addition to the plate method antagonists were added to cultures of Ogawa 40 on the semi-defined medium described by Olitzki & Olitzki (1956), but no inhibition was noted when at least 100 µg. streptomycin/ml. was present. To avoid false interpretation by an inhibitory effect of the anaerobic medium, streptomycin was brought in contact with this medium before the clostridia were inoculated. No inhibitory effect was observed.

In a final experiment (Table 3) it was shown that under the same experimental conditions as used for demonstrating the antagonism, an increase in concentration of streptomycin above the standard concentration of 20 µg./ml. produced less antagonism than at the lower streptomycin concentration. The highly active preparations of *Pseudomonas aeruginosa* required for the complete disappearance of the antagonism 640 µg. streptomycin/ml., while with relatively weak inhibitors from Enterobacteriaceae and Bacillaceae the inhibiting effect disappeared at 40–80 µg streptomycin/ml. These results confirm the observations made in the former experiments where a preparation of *Pseudomonas aeruginosa* diluted 1/16 inactivated 10 µg. streptomycin/ml. It was therefore expected that the undiluted preparation should inactivate at least 160 µg. streptomycin/ml.; Table 3 confirms this expectation. In the presence of the preparation from *Pseudomonas aeruginosa* and 160 µg. streptomycin/ml. there appeared a zone of diameter of 15 mm., indicating that only 5 µg. streptomycin/ml. remained active, and that 155 µg./ml. had been inactivated. On the other hand, it was expected that preparations which even when undiluted inactivated only 10 or 15 µg. streptomycin/ml. would not give any inhibitory effect in the presence of 80 µg. streptomycin/ml. Table 3 confirms this expectation for all the weakly active preparations.

Table 3. *Effect of increasing streptomycin concentrations on the streptomycin-inhibiting effect of different bacterial preparations*

Extract prepared from strain	Diameter of zone of decomposition (mm.) in the presence of streptomycin (µg./ml.)				
	20	40	80	160	640
Pseudomonas hydrophila	14·2	18	21	—	—
P. non-liquefaciens	15	18	20·5	—	—
P. fluorescens	12·2	15	18·5	21	—
Pseudomonas sp. from eggs	15	18·5	21	—	—
P. aeruginosa	0	0	8·5	15	21
Pseudomonas sp. 56/1	0	0	9	15·5	21
Vibrio comma (Ogawa 1)	19·7	20·5	20·5	—	—
Proteus vulgaris V_1	17	20	20·5	—	—
P. morganii M_1	10	16	19	21	—
P. rettgeri R_1	18	20	21	—	—
Escherichia coli	15·5	18	21	—	—
Klebsiella sp.	19	20·5	21	—	—
Shigella flexneri	17	20	21	—	—
Clostridium parabotulinum 3732	15	18·5	20	—	—
C. welchii 8798	15	19	20·5	—	—
C. histolyticum 503	18	19	20	—	—
Bacillus megaterium	15	18·5	21	—	—
B. subtilis	16·5	19	21	—	—

Remarks. Diameter of the zone of decomposition in the presence of 20 µg. streptomycin/ml. without inhibition was 20·7 mm.

REFERENCES

Lightbown, J. W. (1950). An antagonist of dihydrostreptomycin and streptomycin produced by *Pseudomonas pyocyanea*. *Nature, Lond.* **166**, 356.

Lightbown, J. W. (1954). An antagonist of streptomycin and dihydrostreptomycin produced by *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **11**, 477.

Olitzki, A. L. & Olitzki, Z. (1955). Pathogenicity and antigenicity of streptomycin dependent mutants of *Vibrio comma* (types: Inaba and Ogawa). *Exp. Med. Surg.* **13**, 332.

Olitzki, A. L. & Olitzki, Z. (1956). Nutritional requirements of a streptomycin-dependent *Vibrio comma* (Ogawa) and its use for the quantitative determination of streptomycin in urine and serum. *Antibiotic Med.* **2**, 317.

Olitzki, A. L. & Olitzki Z. (1957). The action of streptomycin on the amylase activity of *Vibrio comma*. *Giorn. Microbiol.* **3**, 109.

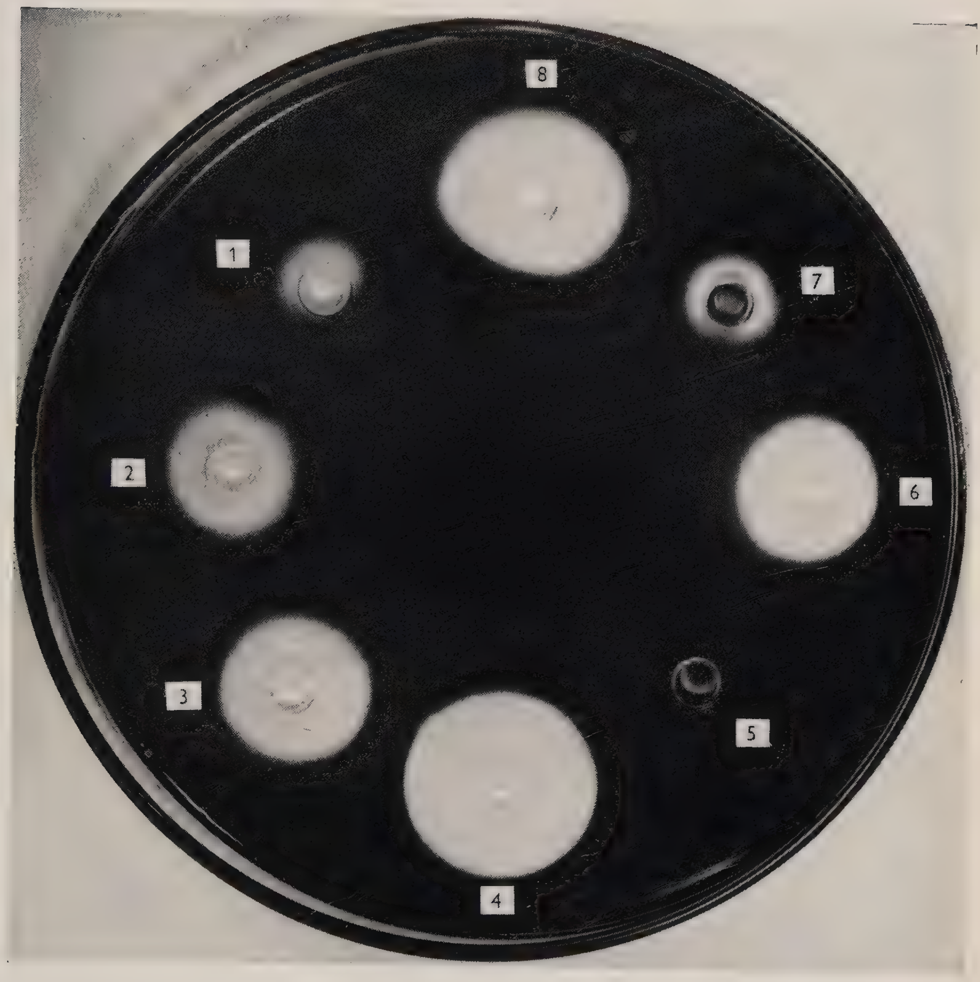
Sulitzeanu, D. & Olitzki, A. L. (1955). Use of a streptomycin dependent strain of *Vibrio comma* for quantitative assay of streptomycin. *Exp. Med. Surg.* **13**, 394.

EXPLANATION OF PLATE

Plate 1. [Natural size]

(1) 2·5 μg. streptomycin/ml. (2) 5·0 μg. streptomycin/ml. (3) 10·0 μg. streptomycin/ml. (4) 20·0 μg. streptomycin/ml. (5) 20·0 μg. streptomycin/ml. + extract of *Pseudomonas* sp. (6) 20·0 μg. streptomycin/ml. + extract of *Bacillus subtilis*. (7) 20·0 μg. streptomycin/ml. + extract of *Proteus morganii*. (8) 20·0 μg. streptomycin/ml. + extract of *Vibrio comma*, strain Ogawa 1.

(*Received* 30 *June* 1958)



A. L. OLITZKI & D. GODINGER—STREPTOMYCIN INHIBITORS. PLATE 1

(*Facing p.* 60)

Cartwright, S. F. & Thorne, H. V. (1959). *J. gen. Microbiol.* **20**, 61–77

Some Applications of Detergents to the Study of the Virus of Foot-and-Mouth Disease

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SUMMARY: The virus of foot-and-mouth disease is highly resistant to the detergents sodium dodecyl sulphate (SDS), cetyl trimethyl ammonium bromide (CTAB) and Tween 80. Results are recorded which show the possibilities of using SDS in the extraction of virus from infected cells, in concentrating virus by elution from adsorbents, and in increasing yields in the filtration and ultracentrifugation of virus suspensions.

Since the work of Burnet & Lush (1940), who found that the viruses of herpes, louping ill, influenza, myxomatosis, psittacosis and vaccinia were inactivated by anionic detergents, there have been few investigations of the effect of synthetic detergents on animal viruses. Only two viruses appear to have been added to those above, those of lymphocytic choriomeningitis (Stock & Francis, 1943) and the Rous sarcoma (Guerritore, 1957, 1958), both also inactivated by anionic and cationic detergents. One of the present authors (H.V.T.) and Miss J. Rogers have observed that the virus of vesicular stomatitis is inactivated by anionic and cationic detergents (unpublished).

Studies of the action of bile salts, naturally-occurring detergents, however, show that inactivation is by no means general. Galloway (1931) obtained results which indicated that the virus of foot-and-mouth disease resisted the action of sodium deoxycholate at concentrations up to 10 % (w/v), and Theiler (1957) showed that although all the arthropod-borne viruses he tested were inactivated by a 1/1000 dilution of this salt, strains of the viruses of poliomyelitis, encephalomyelitis, Coxsackie disease and encephalomyocarditis were resistant at this concentration.

It is shown in the present paper that the virus of foot-and-mouth disease is highly resistant to at least three synthetic detergents, one anionic (sodium dodecyl sulphate) one cationic (cetyl trimethylammonium bromide) and one non-ionic (Tween 80). In addition, some ways are presented in which the resistance to sodium dodecyl sulphate may be exploited in studies of the virus. These might also be of use in the study of other resistant viruses.

METHODS

Virus strains. In most of the work a Ven. 1 Vallée 0 type virus strain originally recovered from an outbreak of the disease in Venezuela in 1950, twice passaged in cattle and subsequently in tissue culture of cattle tongue epithelium was used. The virus suspensions used were of the 125th to 160th passages. For some experiments strains from different sources which had been passaged in cattle, guinea-pigs, mice, eggs and monolayer cultures of pig kidney cells were used.

Virus suspensions. The method used to prepare suspensions of Ven. 1 culture virus was similar to that described by Cartwright, Pay & Henderson (1957). Fragments of tongue epithelial tissue were infected with seed virus and incubated at 37° in sealed flasks containing an atmosphere of 95 % (v/v) oxygen + 5 % (v/v) CO_2, in a medium (15 ml./g. tissue) consisting of Tyrode's solution, 0·5 % (w/v) lactalbumin hydrolysate, 200 mg. dihydrostreptomycin sulphate/ml., 200 units benzylpenicillin (sodium salt)/ml. and 0·001 % (w/v) phenol red. After 18 to 24 hr. the culture liquid, subsequently referred to as supernatant virus suspension, was separated from the infected tissue by centrifugation. Virus filtrates of higher titre, used in most experiments and subsequently referred to as ground tissue suspensions, were prepared by grinding the tissue with carborundum powder and extracting with 2·5–10 ml. 0·04 M-phosphate buffer (pH 7·6)/g. tissue or equal parts of this buffer and Hartley's broth (Hartley's broth + phosphate) and filtering through Seitz EK pads.

The M. 11 culture virus suspensions were the supernatant fluids from infected pig kidney monolayers clarified by centrifugation for 15 min. at 3000 rev./min.

Suspensions of the Ven.1 cattle and guinea-pig strains, the M. 11 mouse strain and the Rho. 1 strain were prepared by suspending the ground infected tissues in phosphate-buffered Hartley's broth and filtering through Seitz EK pads.

Preparation of cell suspensions. Suspensions of cattle tongue epithelial cells were prepared by digestion of fragments of tongue epithelium with trypsin as described by Cartwright *et al.* (1957). Pig kidney cells were grown as monolayers in Pyrex glass bottles (Sellers, 1955) and cell suspensions were obtained by washing once with phosphate-buffered saline (pH 7·6), followed by treatment with 1/5000 di-sodium versenate in the same medium for 30 min. at 37°. The cells were deposited by centrifugation at 1000 rev./min for 5 min. washed with phosphate-buffered saline and finally resuspended in Tyrode's solution containing 0·5 % (w/v) lactalbumin hydrolysate.

Preparation of monolayers of tongue epithelial cells. The monolayers were grown in 10 ml. screw-capped neutral glass bottles from suspensions of epithelial cells as described by Pay (1957).

Titration of virus suspensions. Serial tenfold dilutions were prepared in 0·04 M-phosphate buffer (pH 7·6) and inoculated intra-peritoneally into groups of five or ten unweaned mice, using a 0·04 ml. dose (Skinner, 1951). The virus content expressed as ID 50/ml. was obtained from the 50 % end-point dilution calculated by the method of Spearman-Kärber (Kärber, 1931). This method gives easier calculations and is more amenable to statistical treatment (Finney, 1952) than that of Reed & Muench (1938). The end-points resulting from the use of these two methods differed by less than 0·1 log unit.

The results of titrations of 45 infective filtrates used in the course of the work were analysed and gave standard errors of $\log_{10}$ ID 50/ml. calculated by the method of Irwin & Cheeseman (1939), ranging from 0·17 to 0·4 for

5 mice/dilution and from 0·14 to 0·27 for 10 mice/dilution, with mean standard errors of 0·27 and 0·19, respectively. An experiment in which 12 separate titrations of a single filtrate using 5 mice/dilution were performed gave a standard deviation of 0·22, in agreement with the estimate derived above from a single titration.

In comparing the titres of virus suspensions the difference between the titres of two virus suspensions was considered significant when it equalled or exceeded twice the standard error of the difference.

Detergents. The following detergents supplied by L. Light and Co., Ltd. were used: sodium dodecyl sulphate (SDS), cetyl trimethylammonium bromide (CTAB), Tween 80. The SDS was recrystallized twice from ethanol and extracted with ether and the CTAB was recrystallized twice from acetone although the products as supplied gave indistinguishable results from the purified substances when tested in parallel experiments. All detergents were dissolved in 0·04 M-phosphate buffer (pH 7·6) for use; concentrations throughout are expressed as % (w/v).

Adsorbents. The adsorbents used were Attaclay (a magnesium silicate clay Attapulgite, Attapulgus Co., Ltd., U.S.A.), Celite (Snowfloss, Johns-Manville Co. Ltd.), aluminium hydroxide gel (Alhydrogel Dansk Svovlsyre-og Superphosphat-Fabrik, Copenhagen) and calcium phosphate gel prepared by adding 0·3 M-$CaCl_2 6H_2O$ to 0·2 M-$Na_3PO_4 . 12H_2O$ followed by washing with distilled water until free from chloride.

All adsorbents were washed with 0·04 M-phosphate buffer (pH 7·6) and the excess was removed by centrifugation before addition of virus suspension. The mixture was allowed to stand, with occasional shaking, for 30 min. or longer at 4°, before centrifugation to separate the adsorbent from the liquid.

Protein determination. In some experiments rough estimates of the protein concentration of the solutions were obtained by measurements of the ultraviolet (u.v.) absorption of the sample, diluted when necessary, at 280 mμ, with corrections for media containing lactalbumin hydrolysate or Hartley's broth, by using a Beckmann model DU spectrometer with 1 cm. cells. The optical density (O.D.) was not related to actual protein content because of the heterogenous nature of the material.

Detergent determination. Concentrations of SDS and CTAB were estimated by the method of Barr, Oliver & Stubbings (1948) with bromo phenol blue as indicator.

RESULTS

Resistance of the virus of foot-and-mouth disease to SDS, CTAB and Tween 80

SDS. The effect of SDS on the Ven. 1, Dutch 0/1957, Kenya 3/57 tongue epithelial tissue culture strains, an M. 11 pig kidney culture strain and on a Rho. 1 egg strain (Pan 37) in short periods of treatment at 25° and 37° was investigated. The filtrates were diluted 1/10 in solutions of SDS of 0·05–5·0 % concentration to decrease loss of SDS through binding by non-viral material. No significant decrease in titre was found with any of the strains tested up to

90 min. Experiments over a much longer period (Table 1) with strain Ven. 1 filtrates diluted 1/1000 in detergent solutions of concentration 0·1–5·0 % at 25° showed that even after 48 hr. there was no significant change in infectivity. Serum neutralization tests showed that no change of type specificity occurred, indicating that the virus was antigenically unchanged.

Table 1. *The action of sodium dodecyl sulphate (SDS) on the Ven.* 1 *strain at* 25°

	SDS concentration (%)				
	0·0	0·1	1·0	2·0	5·0
	Virus titre (log ID 50/ml.)				
Time (hr.)					
0·0	5·1	—	—	—	—
6·0	4·9	4·1	5·3	5·5	5·3
24·0	4·9	—	4·9	5·1	4·7
48·0	4·7	—	4·9	4·9	4·9

CTAB. Similar results were obtained with CTAB. No significant decrease in titre was found up to 120 min. with any of the strains tested (a Ven. 1 cattle strain, the M. 11 pig kidney culture strain and the Rho. 1 egg strain (Pan 37)), and up to 20 hr. with the Ven. 1 tissue culture strain (Table 2) with concentrations of 0·05–1·0 % CTAB at 25°. In tissue culture filtrates precipitation occurred at a detergent concentration of about 0·05 %, with the removal of about 25 % of the total u.v. absorption but of less than 1·0 % of the infectivity estimated by titration of the resuspended precipitate.

Tween 80. A few experiments with Tween 80 (non-ionic detergent) gave similar results to the ionic detergents. No decrease in titre was found at 1 % concentration for times up to 20 hr. (Table 3).

Table 2. *The action of cetyl trimethylammonium bromide (CTAB) on the Ven.* 1 *strain at* 25°

	CTAB concentration (%)			
	0·0	0·05	0·2	1·0
	Virus titre (log ID 50/ml.)			
Time (hr.)				
0·0	4·6	—	—	—
2·0	4·9	4·5	4·3	4·9
5·0	5·1	5·0	5·1	4·9
20·0	4·9	4·4	4·3	4·5

Table 3. *The action of Tween* 80 *on the Ven.* 1 *strain at* 25°

	Tween 80 concentration (%)	
	0·0	1·0
	Virus titre (log ID 50/ml.)	
Time (hr.)		
6·0	5·0	4·8
12·0	4·6	5·3
20·0	4·9	5·3

Extraction of virus from infected cell suspensions

Suspensions of tongue epithelial and pig kidney cells were infected with strain Ven. 1 virus, washed with antiserum to remove extracellular virus, and resuspended in Tyrode's + 0·5 % lactalbumin hydrolysate with *c.* 10^6 cells/ml. as described by Cartwright *et al.* (1957). The cell suspensions were incubated at 37° and samples of the whole culture were taken at intervals during the latent and release periods. One portion of each sample was titrated to determine the total infectivity and 1·0 ml. was centrifuged at 1000 rev./min., for 5 min., the supernatant fluid removed and titrated, and the deposited cells treated with 1·0 ml. of a solution of SDS for 10 min. This suspension was centrifuged at 3000 rev./min. for 15 min. and the supernatant fluid titrated.

Table 4. *The extraction of virus from infected cell suspensions*

Extracting medium	Whole culture	Supernatant fluid	Extracted virus
Tongue epithelial cell suspensions 2 hr. after infection			
	Virus titre (log ID 50/ml.)		
0·5 % SDS	5·0	1·7	4·8
1·0 % SDS	5·4	2·0	5·3
0·1 % Tween 80	5·1	2·2	2·2
0·5 % Tween 80			2·7
1·0 % Tween 80			⩽2·1
0·1 % CTAB			3·5
0·5 % CTAB			3·9
1·0 % CTAB			3·6
2·0 M-phosphate buffer (pH 7·6)			<1·9
5·0 M-NaCl (pH 7·6)			<2·7
2·0 M-glycine (pH 7·6)	4·5	<1·4	⩽3·0
Pig kidney cell suspensions 2 hr. after infection			
0·1 % SDS	4·9	2·3	>4·0
0·5 % SDS	4·8	2·3	4·8
1·0 % SDS	4·7	2·3	5·0
0·1 % Tween 80	4·9	2·3	2·7
0·5 % Tween 80			3·3
1·0 % Tween 80			2·9
1·0 % CTAB	4·7	1·9	3·3
0·5 % CTAB			3·7
1·0 % CTAB			3·9
2·0 M-phosphate buffer (pH 7·6)	4·9	2·3	<1·9
5·0 MNaCl (pH 7·6)			4·1
2·0 M-glycine (pH 7·6)	5·1	2·1	2·9

The recovery of infectivity at different stages of multiplication is shown in Tables 4 and 5. Virus was extracted from infected cells in approximate equivalence to the total virus content and depended in amount on the stage of multiplication reached at the time of sampling. Although some extraction occurred with 0·05 % SDS, a concentration of 0·5 % was necessary to effect complete extraction.

None of the following reagents: phosphate, saline, glycine, CTAB, Tween 80, extracted more than about 10 % of the total virus from intact infected cells.

Table 5. *Extraction of virus with 0·5 % SDS from pig kidney cell suspensions throughout the growth cycle*

Time (hr.)	Whole culture	Supernatant fluid	Extracted virus
	Virus titre (log ID 50/ml.)		
1·5	5·1	2·1	4·9
2·5	4·3	2·7	4·7
3·0	5·7	5·3	6·3
4·0	7·3	⩾6·7	⩾7·5
5·0	⩾8·3	6·7	⩾7·9

The extraction of virus from infected cells during the latent period by SDS was of particular interest in view of the results of Cartwright *et al.* (1957) who found that, on the average, less than 1 % of the total virus content was extracted with 0·04 M-phosphate buffer (pH 7·6) after grinding with abrasives. On extracting ground cell debris with 0·5 % SDS titres equal to those of the whole culture were obtained. Since the completion of the work recorded in this paper this virus has been further investigated (Cartwright & Thorne, 1958) and has been shown to be cell-associated virus, resistant to the action of antiserum, but without apparent influence in virus multiplication.

Extraction of virus from infected cell monolayers

Monolayers of tongue epithelial cells were infected and washed as described for cell suspensions. At selected intervals the supernatant fluid was removed and replaced by an equal volume of 0·5 % SDS. After 5 min. this was removed and any suspended cells and debris deposited by centrifugation. Five cultures were used for each time investigated and the liquids were pooled for titration. The results in Table 6 show the increase in virus content with time, and the high ratio of cellular virus-titre to the titre of the virus normally released into the medium.

Table 6. *Extraction of virus with 0·5 % SDS from tongue epithelial cell monolayers throughout the growth cycle*

Time (hr.)	Supernatant fluid	Extracted virus
	Virus titre (log ID 50/ml.)	
1·5	<0·5	2·5
2·0	1·8	2·9
3·0	⩽1·3	⩾5·7
3·5	3·9	⩾6·3
4·0	4·3	⩾6·9

Extraction from infected tissue fragments

The possibility of extending this method to the extraction of virus from tissue fragments used in the production of virus by the method of Frenkel (1947, 1953) was investigated. In these experiments extraction for 30 min. at 37°

with 10–25 ml. 1–2 % SDS solution/g. tissue gave titres of 7·5–8·5 log ID 50/ml. which differed by less than ±0·4 log unit from those obtained by grinding with carborundum. These extracts were clear solutions readily separable by centrifugation from the tissue fragments.

Effects of SDS and CTAB on cells

In the presence of SDS at concentrations 0·5–1·0 % microscopic examination showed that tongue epithelial cells were stained in a few seconds with eosin, indicating cell death (Schrek, 1936); after about 30 sec. marked changes in the appearance of the cells occurred, indicative of cell-wall damage, with the presence in the surrounding fluid of intertwined fibrillar structures, probably of aggregated cytoplasmic material. In suspensions of cells this material and the cell debris aggregated rapidly to form a gelatinous precipitate. Similar effects were observed in pig kidney cells but the cells became completely disrupted with none of the original structure remaining. CTAB in the same concentration range produced staining of tongue epithelial cells by eosin, and in a small proportion of both tongue and pig kidney cells, cytoplasmic protrusions but no extensive disintegration. Tween 80 (1·0 %) produced no microscopically observable effects in the structures of either type of cell. Solutions of 2·0 M-phosphate, 5·0 M-NaCl and 2·0 M-glycine (pH 7·6) caused crenation of the cells, presumably due to osmosis, but no disintegration. These observations indicate that the higher recovery of virus from cells by using SDS, compared with the other solutions tested, was due primarily to its disruptive action on the cells and not to an elution mechanism dependent on electrical charge.

The elution of strain Ven. 1 *tissue culture virus from some adsorbents by SDS*

The investigations have been mainly concerned with the efficiency of elution of strain Ven. 1 tissue culture virus by SDS as compared with other agents, with particular reference to the possibility of concentrating the virus by elution in small volume. The adsorbent Attaclay was used for most of this work, but in some experiments the elution of virus from Celite, calcium phosphate gel and aluminium hydroxide gel was studied. Attaclay was used by Hiatt, Rothstein & Yager (1951) and by Breese & Briefs (1953) to decrease the protein concentration of chick embryo and mouse brain suspensions, in studies of Japanese encephalitis virus and Coxsackie virus, respectively. In both of these studies it was possible, because of the high protein level, to adsorb the greater part of the protein without decreasing the virus titre of the suspensions.

Adsorption. To provide a safeguard against the possibility of inactivation being interpreted as adsorption, the degree of adsorption was determined by the titration of both the adsorbent and the supernatant fluid. In the case of Attaclay it was demonstrated by centrifugation of infective suspensions and titration of the supernatant fluids that complete elution occurred on dilution to 1/1000 in 0·04 M-phosphate buffer (pH 7·6). Since the virus filtrates used had titres greater than 10^6 ID 50/ml. complete elution would occur in the dilutions inoculated and titration of the adsorbent would be expected to give a true estimate of the quantity of adsorbed virus.

All of these adsorbents removed virus from strain Ven. 1 tissue culture fluids to the extent of Attaclay shown in Table 7. The amount of virus adsorbed, given by the 'adsorbent titre', was within the limits of error of titration, in agreement with the amount calculated from the difference in titre between that of the original filtrate and the final supernatant fluid. Although precise estimation of the amount of virus adsorbed was not possible, owing to the inaccuracy of the titration method, it is apparent that a correlation exists between the quantity of virus adsorbed and the amount of adsorbent used. Fifty mg. Attaclay/ml. were adequate to adsorb more than 95 % of the virus from ground tissue filtrates of tissue strength 1/10 to 1/2·5. From 50 to 80 % of the virus was adsorbed by 25 mg. Attaclay/ml., with only slight adsorption by 10 mg./ml. Sedimented volumes of the other adsorbents roughly equal to that of Attaclay adsorbed 95 % or more of the virus from ground tissue filtrates. This amounted to 50 mg. Celite/ml., 25 mg. calcium phosphate/ml., expressed as dry weight, and 7·5 mg. aluminium hydroxide/ml., expressed as Al_2O_3.

Table 7. *The adsorption of virus by Attaclay*

	Adsorbent concentration (mg./ml.)	Initial virus titre	Adsorbent titre	Supernatant fluid titre	Unadsorbed virus (%)
		Virus titre (log ID 50/ml.)			
Ground tissue suspensions (g./ml.)					
1/5	10	8·7	6·9	8·2	34
1/5	25	8·7	8·3	7·1	2
1/5	50	8·7	8·6	6·7	1
1/2·5	25	7·8	—	7·0	14
1/2·5	50	7·8	—	6·1	2
Supernatant virus suspensions	10	7·1	6·5	6·8	50
	16	7·7	—	5·1	1
	50	7·8	—	4·1	$<0·1$

Ultraviolet absorption measurements of virus suspension, filtered through Attaclay deposited as a thin layer on filter-paper in a Buchner funnel, indicated that adsorption of u.v. absorbing material and virus ran parallel and that no significant adsorption of virus occurred after saturation of the adsorbent with the other constituents of the medium. With both types of virus suspension a much greater proportion of virus than of u.v.-absorbing material was adsorbed. In an experiment with ground tissue virus of titre 10^8 ID 50/ml. and o.d. 4·3 at 280 mμ, filtered through 1·0 g. of adsorbent, successive 20 ml. volumes of filtrate had titres of 10^6, $10^{6·9}$, $10^{7·6}$, 10^8 and 10^8 ID 50/ml. with o.d. values of 2·45, 3·16, 3·8, 3·85 and 4·0, respectively; i.e. after the passage of 40–60 ml. adsorption of both virus and u.v.-absorbing material rapidly decreased.

In most cases 10 mg. Attaclay/ml. added to tissue culture supernatant fluids was sufficient to adsorb the greater part of the virus. Two experiments in which supernatant virus was filtered through Attaclay gave the following results: the first with an initial titre $10^{7\cdot8}$ ID50/ml. and O.D. 2·6 gave, for successive volumes of 25, 75 and 25 ml., titres of $10^{4\cdot0}$, $10^{6\cdot6}$ and $10^{6\cdot9}$ ID50/ml. and O.D. values of 0·1, 1·5 and 1·6 respectively, the second with initial titre $10^{7\cdot8}$ ID50/ml. and O.D. 2·2 gave for successive volumes of 70, 50 and 50 ml. titres of $10^{5\cdot8}$, $10^{6\cdot7}$ and $10^{7\cdot5}$ ID50/ml. and O.D. values of 0·3, 1·7 and 1·7, respectively. These results showed that the treatment of approximately 100 ml. of supernatant virus with 1 g. adsorbent resulted in the adsorption of more than 95 % of the virus, followed by an increase in both the titre and optical density of the filtrate. The smaller quantity of Attaclay required for the adsorption of virus from supernatant suspensions is in keeping with the lower protein content as indicated by the optical density.

Elution

Elution from Attaclay with SDS. Adsorbed virus was eluted from Attaclay by solutions of SDS in amounts increasing with the concentration of SDS (Table 8). A concentration of 0·1–0·5 % SDS was found to elute all the adsorbed virus, in a volume equal to that of the original virus suspension at room temperature, in less than 15 min. The concentrations of eluting agents given throughout refer to the concentration of the solution added. In practice the effective concentration is lowered by the liquid adhering to the adsorbent (2 ml./g.). In the case of elution in 1/5 of the original volume the effective concentration is approximately half of the nominal concentration. To obtain concentration with respect to the original virus, i.e. elution in a smaller volume than the initial one, higher concentrations of SDS were required. For these experiments 1·0 ml. of the eluting agent was added to 500 mg. adsorbent, when 50 mg. adsorbent were used/ml. virus suspension. Because of the liquid held by the adsorbent this gave only a five-fold volume concentration factor.

Table 8. *The elution of virus from Attaclay with SDS*

SDS (%)	Adsorbent concentration (mg./ml.)	Initial virus titre	Adsorbent titre	Eluate titre	Volume concentration factor
		Virus titre (log ID50/ml.)			
Ground tissue suspensions					
0·05	50	7·9	7·8	6·7	1
0·10	50	7·9	7·8	7·9	1
0·50	50	7·8	7·8	7·5	1
0·50	50	7·5	7·4	≤7·3	5
1·0*	50	8·1	8·1	8·7	5
		(7·5–8·8)	(7·4–8·7)	(7·8–9·1)	
1·0	25	7·8	7·7	9·1	10
Supernatant suspensions					
1·0	20	7·4	7·1	8·0	8
2·0	10	6·2	6·0	7·3	20

* Mean of eight experiments.

The mean log difference in titre between original virus and concentrate for 8 experiments with 1·0 % SDS was 0·6 (range 0·3–1·1) compared to the expected 0·7 (log 5). In some cases volume concentration factors of 10- and 20-fold were obtained by using 25 and 10 mg. adsorbent/ml. with the virus from ground tissue and supernatant fluids respectively, and *c.* 10-fold concentration of virus was found in these experiments.

Ultraviolet absorption measurements of the concentrates showed that the O.D. value at 280 mμ increased with increasing detergent concentration and in parallel with increasing degree of virus elution. The greater part of the protein remained adsorbed, giving eluates of the same order of optical density as the original suspension and hence with a higher ratio of titre to protein concentration.

Elution from Attaclay with other media. The efficiency of SDS as an eluting agent was compared with that of various solutions buffered at pH 7·6. Neither CTAB nor Tween 80 at concentrations up to 1·0 % eluted virus in quantities in excess of 5 % of that adsorbed. Similar results were obtained with glycerol (20–100 %, v/v) which was found by Rushchits (1954) to be effective in eluting the virus of foot and-mouth disease from calcium sulphate. Glycine at concentrations greater than 1·5 M eluted the greater part of the adsorbed virus; concentration of virus was achieved, but with a recovery of only 30 %. Solutions of 1·0 M-NaCl eluted only 10–25 % of the virus and this decreased markedly when concentration of the virus was attempted at salt concentrations up to 5·0 M. Almost complete elution was achieved with phosphate buffers (pH 7·6) at 0·75–1·25 M; at concentrations less than or equal to 0·5 M less than 1 % of the virus was eluted; at concentrations higher than 1·5 M, where precipitation of some of the protein of the medium occurred with adsorption of virus, elution was negligible. Only 10–20 % of the virus was recovered when five-fold concentration was attempted with 1·0 M-phosphate buffer (pH 7·6), obtained by the addition of 2·0 M-phosphate to the adsorbent.

Elution from aluminium hydroxide gel, calcium phosphate gel and Celite. Good recoveries of virus were obtained from Celite and aluminium hydroxide with SDS at 0·5 % (Table 9). It was expected from the insolubility of calcium dodecyl sulphate that low concentrations of SDS would be ineffective in eluting virus from calcium phosphate gel; however, elution in excess of 10 % occurred with 1·0 and 5·0 % solutions of SDS (Table 9).

Elution of virus from aluminium hydroxide with a degree of concentration comparable to that obtained with SDS occurred with 2·5 and 5·0 M-NaCl. Elution from calcium phosphate comparable to that by SDS from Attaclay resulted from the use of phosphate buffers (pH 7·6) at 0·5–1·5 M; higher strengths caused precipitation of virus and negligible elution. Concentration was achieved at an effective phosphate concentration of 1·0 M. Efficient elution and concentration were also found with 2·5 and 5·0 M-NaCl. Full recovery of virus from calcium phosphate and aluminium hydroxide resulted from elution with 2·0 M-glycine in a volume equal to that of the original suspension, but not in a smaller volume. Neither Tween 80 (1 %) nor CTAB (1 %) eluted more than 1 % of the virus from these two adsorbents.

Table 9. *Elution of virus with SDS from aluminium hydroxide gel, calcium phosphate gel and Celite*

SDS (%)	Initial virus titre	Adsorbent titre	Eluate titre	Volume concentration factor
	Virus titre (log ID 50/ml.)			
Aluminium hydroxide gel				
0·5	8·2	7·9	7·9	1
2·0	8·2	8·0	8·5	5
Calcium phosphate gel				
0·5	7·5	7·1	⩽5·7	1
1·0	8·3	7·6	7·7	1
5·0	7·6	7·8	7·7	1
5·0	8·3	7·6	8·3	5
Celite				
1·0	8·5	8·5	8·7	1

The use of SDS in the filtration of virus suspensions

A difficulty sometimes experienced with the virus of foot-and-mouth disease is the loss by adsorption on filtration through Seitz EK sterilizing pads. This is particularly severe with the suspensions of low protein content from tissue culture. SDS was used in an attempt to diminish these losses.

Ven. 1 strain suspensions from ground tissue were diluted 1/50 in protein-free media, Hartley's broth + phosphate, amino-Tyrode's solution or Tyrode's solution + 0·5 % (w/v) lactalbumin hydrolysate, to provide conditions of low protein concentration and thus to increase loss of virus. Ten ml. portions were filtered with negative pressure through Seitz EK pads first treated with 0·04 M-phosphate buffer (pH 7·6). The filtrates were collected and titrated. Only when the medium contained lactalbumin hydrolysate was the recovery of virus greater than 0·1 % of the original. The results in Table 10 demonstrate the improvement in titre, in many cases 1000-fold, when SDS was included in the suspensions to be filtered or when a solution of this detergent was passed through the filter after the passage of the virus suspension. Failure

Table 10. *The use of SDS in the filtration of Ven.* 1 *strain virus suspensions*

Suspension medium	SDS (%)	Initial virus titre	Filtrate titre	Eluted titre
		Virus titre (log ID 50/ml.)		
Detergent passed through filter after virus suspension				
Hartley's broth + phosphate	0·1	6·4	2·9	5·7
Amino-Tyrode's	0·5	7·3	⩽2·9	5·5
Lactalbumin hydrolysate	0·5	6·5	5·7	6·8
Detergent mixed with virus suspension to be filtered				
Hartley's broth + phosphate	0·1	6·4	5·4	—
Amino-Tyrode's	0·5	7·0	6·9	—

to recover virus completely in some of the experiments can probably be explained in the first method by the adsorption of SDS by the protein of the medium with a consequent decrease in the effective elution concentration, and in the second by the speed of elution which probably also determined the recovery. In both cases higher concentrations of detergent improved recoveries. Only a small recovery resulted from using glycine and no recovery was obtained when CTAB was used in place of SDS. These results were in accordance with those already given concerning elution by these two reagents.

Experiments with undiluted M.11 tissue culture virus (in a medium consisting of Earle's saline, yeast extract and lactalbumin hydrolysate) gave similar results to those with Ven. 1 virus. Virus in this medium has been found (Mr R. F. Sellers, private communication) to be particularly susceptible to losses through adsorption. The recovery of M.11 virus from mouse tissue was increased 10–50-fold by the use of SDS.

The use of SDS in the centrifugation of virus suspensions

Although ultracentrifugation is a convenient method of concentrating the virus from volumes less than *c.* 1 l., the recoveries are usually only of the order of 30–50 % (Dr C. J. Bradish, private communication). It appeared probable that losses occurred through adsorption of virus on large particle aggregates produced by deposition and discarded in the normal clarification cycle. The practicability of using SDS rather than 0·04 M-phosphate buffer (pH 7·6), the normal suspending medium, was therefore investigated. Ven. 1 strain tissue culture virus was passaged in guinea-pigs and a phosphate-buffer extract of the ground pads clarified by centrifugation to provide starting material. This was centrifuged at 35,000 rev./min. for 50 min. in a Spinco L40 rotor to sediment the virus (Bradish, Brooksby, Dillon & Norambuena, 1952). The deposited pellets were suspended in a small volume of 0·04 M-phosphate buffer (pH 7·6) and to samples of this suspension, containing non-dispersed aggregates of cell debris of a microsomal character, solutions of SDS, glycine and sodium chloride were added to give a three-fold volume concentration factor. After 30 min, at room temperature the suspensions were clarified (3000 rev./min. for 1 hr.) and the supernatant fluids removed for titration. A similar experiment with Ven. 1 tissue-culture virus, using a 12-fold concentration factor, gave much smaller deposits than guinea-pig virus. The results of a typical guinea-pig virus experiment (Table 11) demonstrate the superiority of 1 % SDS over the other solutions used. The recovery from tissue culture suspensions, although greatest with SDS was also good with the other solutions probably because of the small size of the deposit.

Removal of SDS

In many problems the presence of SDS in virus suspensions is undesirable (e.g. in seed virus for tissue culture), therefore methods for its removal are required. Dialysis, electro-dialysis and deposition of the virus by centrifugation should be readily applicable, without infectivity loss, to small volumes. Several other methods have been investigated.

(1) A single extraction with an equal volume of butanol decreased the concentration of SDS in extracts and concentrates from about 1·0 % to less than 0·02 % without loss of virus, and appears to be a suitable and rapid means for use with small volumes, particularly when it is desirable to decrease the protein concentration at the same time.

Table 11. *The recovery of virus from centrifuged deposits*

	Ven. 1 guinea-pig strain	Ven. 1 T.C. strain
	Virus titre (log ID 50/ml.)	
Titre of original virus suspension	6·6	8·0
Titre of deposit	7·2	9·1
	Supernatant from clarification	
Suspending medium		
0·04 M-phosphate buffer (pH 7·6)	6·6	8·8
0·1 % SDS	6·5	8·8
1·0 % SDS	7·3	9·2
1·0 M-glycine	6·4	8·8
0·5 M-NaCl	6·4	8·8

(2) Cooling to 0–4° and removal of the precipitated SDS by filtration or centrifugation has been found to decrease SDS concentrations of the order of 1·0 % to about 0·2 %.

(3) Concentrations less than 0·5 % SDS were decreased to zero by the addition of CTAB in an amount sufficient to precipitate all SDS without loss of virus. Removal of quantities of SDS greater than 1·0% incurred loss of virus by adsorption on the precipitate. This procedure has the advantage that the exact quantity of CTAB necessary can be determined by titration of a sample by the method of Barr *et al.* (1948).

(4) The precipitation of SDS as the insoluble barium salt caused loss of virus by adsorption.

Methods (2) and (3) used in conjunction with one another would seem to be the most suitable, so far investigated, for use with large volumes of virus suspensions.

DISCUSSION

Resistance to detergents might be a general property of the virus of foot-and-mouth disease, independent of its source and type, since the strains tested had been passaged in a variety of hosts, cattle, guinea pigs, mice, eggs or in tissue culture. The resistance to ionic detergents enables some tentative conclusions to be reached about the structure of the virus. In the first place this resistance, considered along with the long established resistance of the virus to the lipid-extracting solvents ether, acetone, ethanol (e.g. Pyl, 1951) and to butanol, suggests that the virus contains no lipid, at least in its outer structure. (It is possibly significant that at least two of the viruses found to be inactivated by SDS by Burnet & Lush (1940), namely influenza and vaccinia, contain lipid; there may be a general correlation between susceptibility to solvents and to detergents whether synthetic or natural.) Further, since detergents denature proteins primarily by disruption of hydrogen bonds and salt linkages, it is possible that linkages of this type do not play a significant role in the structure of the protein of the virus of foot-and-mouth disease. If linkages of protein to nucleic acid occur, these would be expected to be of a co-valent rather than electro-valent character.

The ease and rapidity with which the virus of foot-and-mouth disease can be extracted from the cells by SDS, together with the instantaneous cessation of multiplication, indicates the use that may be made of this reagent in studies of virus multiplication. The total virus content of cells may be estimated when it is impracticable or inconvenient to extract the virus by grinding, for example, from single cells or monolayers. Further, the removal of monolayers from glass by treatment with trypsin or di-sodium versenate is unnecessary, thus decreasing the risk of loss of virus.

The elution of virus from Attaclay in high dilutions of phosphate buffer suggests that in the experiments of Cartwright *et al.* (1957), the virus from the cell debris fraction was probably free at the dilutions used and not bound in any special manner as suggested by these authors. The elution of virus by SDS does not give direct evidence on this point since extensive cell damage occurs and any linkage of a special kind would probably be broken. However, the close correspondence of the titres of the SDS extracts and ground cells indicates that the dissociation of these bonds yields virus which does not differ in infectivity from the normal. Hence there seems to be no particular reason for assuming that the virus is bound strongly to any cell constituent. It is probable that the low infectivity of phosphate extracts from debris was due to the small volume of buffer used to extract and the high ratio of adsorbent (debris) to virus. The plaque-counting method, however, may reveal differences not apparent from the present work.

Although tissue culture methods have greatly facilitated the production of high titre virus, the titre is still, in general, too low for many purposes without concentration. In recent years methods have been developed for the concentration of polio virus from large volumes of tissue culture fluids (Schwerdt & Schaffer, 1955) but in this case the very favourable stability properties of the virus have enabled co-precipitation with the proteins of the medium at pH 4·0–4·5 to be used as an initial concentration step. The extreme lability of the virus of foot-and-mouth disease at low pH values precludes the use of this method, and the methods at present being used for the concentration of this virus (precipitation with ammonium sulphate, ethanol or acetone after deproteinization with chloroform, Pyl & Röhrer, 1955; or protamine, Hansen & Holm, 1950; and ultracentrifugation) although useful for concentration from sources of low volume are not well suited for use on a large scale. It is therefore desirable for this purpose to find a method using either precipitation or adsorption followed by elution. Apart from early reports of elution from adsorbents (Pyl, 1931) before quantitative titration methods were developed, elution of inactivated virus from aluminium hydroxide vaccines with 5% ammonium phosphate and saturated di-sodium phosphate was reported by Hobohm & Petermann (1949) and Hobohm, Petermann & Forrest (1951).

However, the high pH value of these reagents would probably be inimical to live virus, and in any case to obtain concentration of virus it was necessary to follow elution by pervaporation and precipitation with ammonium sulphate. The eluting properties of SDS indicate that its use with adsorbents of greater capacity or with virus suspensions of lower protein content should yield useful degrees of concentration. Although solutions of saline and pH 7·6 phosphate buffer have been found to produce concentrations of the same order, the lower concentrations of SDS required, together with the ease of its removal, recommend its use.

Since neither CTAB nor Tween 80 was found to be an effective eluting agent it is evident that the eluting property of sodium dodecyl sulphate does not derive directly from its surface activity. Elution probably results from the complexing of SDS with the adsorbed protein and virus to the point when repulsion resulting from the high negative charge breaks the bonds with the adsorbent and releases protein and virus into the liquid. The quantity of SDS required for complete elution would then, as found, depend on the amount of protein adsorbed.

No insurmountable difficulties should arise in the use of SDS in the remaining two applications. When virus is being passaged from one animal host to another, the use of SDS in filtration, possibly following its use in extracting tissue, might well be adopted as a routine procedure which should decrease the number of experimental failures resulting from filtration losses in this type of work. In tissue culture passaging, however, the action of SDS on cells would require that its concentration in the inocula was below a harmful level; there is no doubt that this can be effected. Where virus is to be used for vaccines or for experimental investigations, the procedures can again be of value in reducing losses.

While the greater recoveries obtainable in centrifugation should be of value in complement-fixation and other serological studies actual purification of virus may be impaired by the nature of the elution process. This might result from the dispersion of large particles, normally discarded as aggregates, which might through the action of SDS, persist with virus in subsequent stages. However, it is also possible that the action of SDS would be to dissociate the aggregates to components of low sedimentation constant which would be discarded in the supernatant from deposited virus. Apart from these applications which have been investigated, the antibacterial properties of SDS and CTAB might be of value in work with the virus of foot-and-mouth disease.

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REFERENCES

Barr, T., Oliver, J. & Stubbings, W. V. (1948). The determination of surface active agents in solution. *J. Soc. chem. Ind., Lond.* **67**, 45.

Bradish, C. J., Brooksby, J. B., Dillon, J. F. & Normabuena, M. (1952). Ultracentrifugal studies of the infective and complement-fixing components in the virus system of foot-and-mouth disease. *Proc. Roy. Soc.* B, **140**, 107.

Breese, S. S. Jr. & Briefs, A. (1953). Certain physical properties of Coxsackie strains. *Proc. Soc. exp. Biol., N.Y.*, **83**, 119.

Burnet, F. M. & Lush, D. (1940). The action of certain surface-active agents on viruses. *Aust. J. exp. Biol. med. Sci.* **18**, 141.

Cartwright, S. F., Pay, T. W. F. & Henderson, W. M. (1957). Multiplication of the virus of foot-and-mouth disease in culture. *J. gen. Microbiol.* **16**, 730.

Cartwright, S. F. & Thorne, H. V. (1958). The use of two properties of the virus of foot-and-mouth disease in studies of its multiplication. *Nature, Lond.* **182**, 717.

Finney, D. J. (1952). *Statistical method in biological assay.* London: Charles Griffin and Co. Ltd.

Frenkel, H. S. (1947). La culture du virus de la fièvre aphteuse sur l'epithélium de la langue des bovidés. *Bull Off. int. Epiz.* **28**, 155.

Frenkel, H. S. (1953). Research on foot-and-mouth disease; the cultivation of the virus on a practical scale on explantations of bovine tongue epithelium. *Bull. Off. int. Epiz.* **39**, 91.

Galloway, I. A. (1931). Action of sodium taurocholate, sodium glycocholate and sodium desoxycholate on the virus of foot-and-mouth disease. *4th Progress Report, Foot-and-Mouth Disease Committee* (1931) Appendix 3, p. 313, London: H.M.S.O.

Guerritore, D. (1957). Die Inaktivierung des Rous-Sarkoms mittels eines kationischen Detergens. *Z. Krebsforsch.* **61**, 649.

Guerritore, D. (1958). Reversibility of the inactivation of Rous Sarcoma extracts by detergents. *Nature, Lond.* **181**, 419.

Hansen, A. & Holm, P. (1950). Combination of foot-and-mouth disease virus, albumen and protamine. *Acta path. microbiol. scand.* **27**, 882.

Hiatt, G. W., Rothstein, N. & Yager, R. H. (1951). An adsorption technique for the partial purification of Japanese encephalitis virus in chick embryo tissue suspensions. *Proc. Soc. exp. Biol., N.Y.* **78**, 703.

Hobohm, K. O. & Petermann, H. G. (1949). Concentration and demonstration of the complement-fixing antigen in foot-and-mouth disease vaccine type Riems. *Gac. vet., B. Aires* **11**, 56.

Hobohm, K. O., Petermann, H. G. & Forrest, G. E. (1951). Elution and demonstration of the complement-fixing antigen in adsorbed foot-and-mouth disease vaccines. *Gac. vet., B. Aires* **13**, 163.

Irwin, J. O. & Chesseman, E. A. (1939). On an approximate method of determining the median effective dose and its error in the case of a quantal response. *J. Hyg., Camb.* **39**, 574.

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. exp. Path. Pharmak.* **162**, 480.

Pay, T. W. F. (1957). The propagation of the virus of foot-and-mouth disease in monolayer cultures of adult bovine tongue epithelium. *Proc. R. Soc. Med.* **50**, 919.

Pyl, G. (1931). Adsorptionsversuche mit Maul-und-Klauenseuche-Virus in Pufferlösungen. *Zbl. Bakt.* (1. Abt.), **102**, 284.

Pyl, G. (1951). Versuche zur Reinigung des neurotrop modifizierten Meerschweinchen-B-Stammes des Maul-und-Klauenseuche-Virus. *Exp. Veterinärmed.* **4**, 9.

Pyl, G. & Röhrer, H. (1955). Méthodes d'extraction et de concentration du virus aphteux. *Bull. Off. int. Epiz.* **43**, 597.

Reed, L. T. & Muench, H. (1938). A simple method of estimating fifty per cent end-points. *Amer. J. Hyg.* **27**, 493.

Rushchits, A. S. (1954). Purification and concentration of foot-and-mouth disease virus from vesicle fluid. *Trud. Inst. Vet. Alma-Ata*, **6**, 190.

Schrek, R. (1936). A method for counting the viable cells in normal and in malignant cell suspensions. *Amer. J. Cancer*, **28**, 389.

SCHWERDT, C. E. & SCHAFFER, F. L. (1955). Some physical and chemical properties of purified poliomyelitis virus preparations. *Ann. N.Y. Acad. Sci.* **61**, 740.

SELLERS, R. F. (1955). Growth and titration of the viruses of foot-and-mouth disease and vesicular stomatitis in kidney monolayer tissue cultures. *Nature, Lond.* **176**, 547.

SKINNER, H. H. (1951). Propagation of strains of foot-and-mouth disease virus in unweaned white mice. *Proc. R. Soc. Med.* **44**, 1041.

STOCK, C. C. & FRANCIS, T. JR. (1943). The inactivation of lymphocytic choriomeningitis by soaps. *J. exp. Med.* **77**, 323.

THEILER, M. (1957). Action of sodium desoxycholate on arthropod-borne viruses. *Proc. Soc. exp. Biol., N.Y.* **96**, 380.

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Amino Acid Requirements for Formation of the Reductive Amination System in *Bacillus subtilis*

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SUMMARY: *Bacillus subtilis*, grown on nutrient broth, casein hydrolysate, or on a mixture of amino acids simulating casein hydrolysate, yielded washed suspensions with an active reductive amination system. On simple defined media the activity of the organisms was relatively low. Additions of a variety of amino acids, singly or in simple mixtures, yielded suspensions with activities up to about half of that attained with complex media. The performance of 18 amino acids in about 200 different combinations was analysed statistically; no amino acid showed marked and significant stimulation of enzyme formation. It was concluded that a high enzyme yield requires a medium with a complex amino acid relationship, and that the performance of complex media cannot be explained directly in terms of a few active components.

Knowledge of the nutritional factors required for development of specific functions in micro-organisms is very limited, despite the detailed information available concerning the growth-requirements of many species. Not infrequently simple media which will permit growth yield organisms deficient in certain enzymes (Gale, 1940; Wainwright, 1950; Billen & Lichstein, 1950, 1951; Pinsky & Stokes, 1952). Washed suspensions of *Bacillus subtilis* can synthesize a number of amino acids by 'reductive amination' (notably alanine) when incubated with the appropriate keto acid and ammonia (Fairhurst, King & Sewell, 1956; Shah, King, Hollis & Fairhurst, 1957). The organism grows well on simple defined media but the activity of the reductive amination system is much less than in cultures grown on nutrient broth or casein hydrolysate. Sewell & King (1955) found that an artificial mixture of 18 amino acids yielded organisms of the same order of activity as casein hydrolysate but simultaneous omission of threonine and isoleucine decreased the activity almost to that obtained with the basal medium. The organism was therefore capable of synthesizing all the amino acids necessary for growth but apparently required an external source of certain amino acids for optimal development of this particular enzyme system. The present paper describes an attempt to define this requirement.

METHODS

Bacillus subtilis 4 (Fairhurst *et al.* 1956), maintained on nutrient agar slopes, was used throughout.

Amino acids. L-Isomers were used except for glycine, DL-serine, DL-threonine, DL-ornithine and DL-methionine. Purity was checked by paper chromatography. A few experiments with L-threonine and L-methionine suggested that the DL-mixtures could be used without risk of specific inhibitory action on growth or enzyme formation.

Media. The basal medium contained (g.); KH_2PO_4, 0·5; $Na_2HPO_4.12H_2O$, 1; NaCl, 1·25; $(NH_4)_2SO_4$, 2; glutamic acid, 5; tap water to 1 l.; adjusted to pH 7 with NaOH and autoclaved for 15 min. at 15 lb./sq.in. Sucrose, lactate, glycerol, or asparagine could be used as carbon sources alternative to glutamate, but the latter was preferred as it gave better and more consistent growth. Carbohydrate media were also avoided because of the risk of interaction with amino acids during autoclaving. Sucrose, too, was known to have an inhibitory action in the formation of the reductive amination system in broth media (Sewell, 1954).

Supplementary amino acids were added to the basal medium as required equivalent to 0·02 % of the L-isomer. This concentration was chosen for the following reasons. (1) 0·1 % (w/v) of casein hydrolysate added to the basal medium gave almost maximal stimulation of enzyme formation. Presumably, therefore, no individual amino acid would be required above 0·02 % (w/v). (2) Experiments with leucine and methionine showed that the stimulatory action of these amino acids had already reached its maximum at 0·02 % (w/v). (3) High concentrations of the amino acids were undesirable as many of them tended to inhibit growth. (4) Lower concentrations would have led to difficulties, in some cases, through exhaustion of the amino acid before completion of the 36 hr. growth period. In a considerable number of experiments single-dimensional paper chromatograms were run at the end of the growth period to see whether any of the amino acids had been exhausted.

Acid hydrolysate of casein (Oxo Ltd., London), 0·5 % (w/v) added to basal medium where required.

Growth. The amount of organism in cultures and washed suspensions was determined turbidimetrically with the EEL photoelectric colorimeter (Evans Electroselenium Ltd.) which was calibrated by dry-weight determinations (Sewell, 1954). Results are expressed as mg. dry wt. organism/ml.

Preparation of washed suspensions. Roux bottles containing the medium under test (150 ml.) were inoculated with *c.* 0·1 ml. of a 24 hr culture of the organism grown on basal medium. After incubating for 36 hr. at 37° the organisms were centrifuged down in glass tubes (20 min. at 2000 *g*), washed twice by resuspending in distilled water and centrifuging, and finally suspended in a suitable volume of buffer to give an optical density equiv. 6–8 mg. dry wt./ml. The buffer was prepared by mixing 0·2 M-KH_2PO_4 and 0·2 M-Na_2HPO_4 in the proportions required to give pH 7, and diluting to 0·1 M total phosphate. Choice of incubation time was important since Fairhurst *et al.* (1956) found that reductive amination activity decreased very sharply towards the end of the logarithmic phase of growth. The use of a constant incubation-time with different media giving rise to different rates of growth might therefore produce changes in enzyme activity arising solely from differences in the 'physiological age' of the cultures when harvested. Ideally the growth curve of the organism should be determined for each medium used and cultures harvested at the same stage of development. This was done in a few cases but the value of the procedure was doubtful since the curves varied somewhat in form and it was difficult to decide the incubation times required to produce cultures of strictly comparable physiological age. Forunately, however, the 'age of culture' effect proved to be very much less marked on simple media. Both the basal medium itself and a medium containing glutamic acid, leucine, methionine and histidine were examined from this point of view and it was found that the Q_{alanine} values obtained at 36 hr. were only about 20 % below those obtained after 12 hr. of incubation, when growth had only just reached the stage of appreciable turbidity. Thirty-six hr. was chosen as a standard incubation period, since in most cases this gave a reasonable yield of organisms still in the active phase of growth; maximum turbidity was usually reached only after 3–5 days on the defined media. In some of the earlier experiments (Table 1) a 24 hr. incubation period was used; with very complex amino acid mixtures it was necessary to harvest the organisms after 15–20 hr.

Measurement of enzyme activity (Fairhurst *et al.* 1956): buffered bacterial suspension (2 ml.), 0·5 M-sodium pyruvate (1 ml.) and 0·25 M-ammonium sulphate (1 ml.) were incubated together (4 hr. at 37°). Occasionally the organisms tended to settle to the bottom of the tube and were resuspended from time to time by gentle mixing. The reaction was stopped by adding 2 N-H_2SO_4 (2 ml.) and centrifuging down the organisms; the supernatant fluid was then stored at 0°, pending analysis. Blanks were run by incubating the buffered bacterial suspension and the substrates separately and mixing immediately before addition of the acid. The supernatant fluids were examined by single-dimensional paper chromatography to confirm that alanine was the only amino acid present, and the amino-N determined by the Van Slyke nitrous acid method after removal of excess ammonia by boiling with alkali. The activity of the system is expressed in terms of Q_{alanine} after deduction of the blank, which was negligible in most cases.

In each experiment the organism was grown on basal medium for 24 hr. and this culture used to inoculate two Roux bottles containing the medium under test. The bottles were incubated and harvested as described above, a separate suspension made from each bottle, and the Q_{alanine} value determined for both replicate suspensions. Results cited are means of a pair of replicates, or, in a few cases, the means of larger groups. Since (see below) the standard deviation (S.D.) for a single determination under these conditions was 9·5 % it was considered desirable to repeat experiments in which the difference between the replicate pair of readings exceeded 20 %.

RESULTS

Variance between replicates. When replicate determinations of Q_{alanine} were made on the same washed suspension the standard error (S.E.) of a single reading was 5·1 %. When replicate suspensions were prepared simultaneously S.E. of a single determination of Q_{alanine} was 9·5 %, or 6·6 % for the mean of a pair of readings. When replicate suspensions were prepared on different days (which meant, of course, that the same inoculum could not be used for both) the s.e. rose to 18·6 % for a single determination or 13·3 % for the mean of a pair of determinations. A change of at least 25 % was thus required before it could be claimed that the addition of any component to the medium had influenced the activity of the enzyme system. A further complication was the occasional recording, with simple media, of Q_{alanine} values approaching those normally attained only with complex media. These abnormal results were obtained on about a dozen occasions in a series of over 200 experiments, and in all cases a lower value was obtained when the experiment was repeated. As, however, these freak results were obtained in both members of the replicate pair of suspensions they could not easily be dismissed as arising from experimental error. The development of the reductive amination system may depend critically on a number of factors, some of which are not known (cf. Fairhurst *et al.* 1956), and the interpretation of the effects of various additions to the medium requires great caution.

The enzyme is not adaptive. Ammonium pyruvate did not enhance activity significantly when added to either nutrient broth or basal medium.

Influence of amino acids on enzyme formation

The basal medium gave suspensions with average $Q_{\text{alanine}} = 8$. On complex media (nutrient broth, acid hydrolysed casein) values of the order of 50 were obtained after 15 hr. growth. Q_{alanine} values only slightly below those given by casein hydrolysate were obtained when the latter was replaced by a mixture of amino acids prepared in accordance with the known composition of casein (Gordon, Semmett, Cable & Morris, 1949), or by basal medium supplemented with alanine, arginine, aspartic acid, cysteine, glycine, histidine, isoleucine, eucine, lycine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Since the results obtained with complex media could thus be replaced by a 'complete' mixture of amino acids, the effect of adding each available amino acid in turn to the basal medium was investigated (Table 1). Many of the amino acids stimulated enzyme formation, in

Table 1. *Changes in reductive amination activity on adding single amino acids to basal medium*

The organism was grown for 24 hr. at 37° in the basal medium + the amino acid indicated, at concentration equivalent 0·02 % (w/v) of L-isomer. Washed suspensions were then prepared and their enzyme activity (Q_{alanine}) determined.

Addition	Q_{alanine} value	Addition	Q_{alanine} value
None	8	Methionine	24
Alanine	18	Ornithine	15
Arginine	19	Phenylalanine	18
Aspartic acid	17	Proline	5
Cysteine	21	Serine	27
Glycine	17	Threonine	20
Histidine	12	Tryptophan	14
Isoleucine	7	Tyrosine	11
Leucine	22	Valine	26
Lysine	16		

some cases up to half the value obtained with the complex media. The interpretation of the results is made difficult by the fact that many of the amino acids severely inhibited growth. The figures given in Table 1 thus represent, in some cases, cultures still in a very early stage of growth and we cannot be certain that the figures have not been influenced by 'age of culture' effects, especially as in this series of experiments the cultures were harvested after only 24 hr.

Various mixtures of those amino acids which appeared to be most stimulatory were now prepared in an attempt to achieve higher activity. Over 200 such combinations were tried, mostly comprising groups of from 3 to 7 amino acids. These results were difficult to interpret since the performance of a given amino acid was found to be much influenced by the presence of other amino acids in the medium. The results with lysine (Table 2) illustrate this. It neither stimulated nor retarded growth when added to the basal glutamate medium (Expt. 1), but the enzyme activity was increased from 8 to 16 units. There was also an appreciable increase on adding lysine to the relatively simple media 4*a*, 5*a* and 7*a*; but it depressed activity in the combinations 2*a*, 3*a* and 6*a*. With the more complex media similar irregularity was found. Thus medium 8*a* yielded suspensions with $Q_{\text{alanine}} = 30$ and this relatively high value was not affected by the further addition of serine (9*a*). But while addition of lysine had no effect on the performance of the serine-containing medium (9*b*) it severely depressed the formation of the enzyme in the corresponding no-serine mixture 8*b*. The action of lysine cannot thus be determined by simply

Table 2. *Effect of lysine on enzyme production*

The organism was grown at 37° on basal medium (which included L-glutamic acid, 0·5%) with addition of other amino aci as indicated, at 0·02% of L-isomer. After 36 hr., the amount of growth (culture density) was estimated, the organisms harvest and the enzyme activity (Q_{alanine}) determined. Experiments are grouped in pairs; the second member of each pair (*b*) bei identical with the first (*a*), except for the addition of lysine. The difference between the pair of Q_{alanine} values thus records t increase or decrease in enzyme activity following addition of lysine to the medium in question.

Expt.	Glutamic acid	Leucine	Methionine	Glycine	Serine	Threonine	Alanine	Arginine	Isoleucine	Histidine	Lysine	Q_{alanine}	Difference due to lysine (+ or −)	Grow (mg. ml. $\times 10^2$
1*a*	+	.	.	.	.	.	.	.	.	.	.	8	+8	33
1*b*	+	.	.	.	.	.	.	.	.	.	+	16		29
2*a*	+	+	.	.	.	.	.	.	.	.	.	22	−6	27
2*b*	+	+	.	.	.	.	.	.	.	.	+	16		80
3*a*	+	.	+	.	.	.	.	.	.	.	.	24	−7	30
3*b*	+	.	+	.	.	.	.	.	.	.	+	17		70
4*a*	+	.	.	+	.	.	.	.	.	.	.	17	+5	7
4*b*	+	.	.	+	.	.	.	.	.	.	+	22		17
5*a*	+	.	.	.	.	.	.	+	.	.	.	19	+3	7
5*b*	+	.	.	.	.	.	.	+	.	.	+	22		13
6*a*	+	+	+	.	.	.	.	.	.	.	.	22	−5	27
6*b*	+	+	+	.	.	.	.	.	.	.	+	17		30
7*a*	+	.	.	.	+	+	.	.	.	.	.	15	+5	23
7*b*	+	.	.	.	+	+	.	.	.	.	+	20		8
8*a*	+	+	+	.	.	.	+	.	.	+	.	30	−20	27
8*b*	+	+	+	.	.	.	+	.	.	+	+	10		14
9*a*	+	+	+	.	+	.	+	.	.	+	.	30	0	12
9*b*	+	+	+	.	+	.	+	.	.	+	+	30		12
10*a*	+	+	+	.	.	.	+	.	+	+	.	17	−7	10
10*b*	+	+	+	.	.	.	+	.	+	+	+	10		10

adding it to an arbitrarily-chosen medium. Quite apart from some of the factors discussed under 'variance between replicates', it can appear as either a stimulator or as an inhibitor of enzyme formation, according to the medium selected for testing purposes. We therefore tried to assess its influence by grouping the experiments in pairs as in Table 2; this shows that incorporating lysine into the 10 different media decreased enzyme formation, on an average, by 2·4 Q_{alanine} units, but the value of P (the probability of the decrease being due to chance alone) is 0·4, so the results may well be fortuitous and cannot serve as a basis for any claims that lysine has consistent and demonstrable effect on enzyme production.

Table 3. *Effect of amino acids on enzyme formation*

Pairs of media were examined, the members of each pair differing only in the presence or absence of the amino acid listed in col. 1. The number of such pairs examined in each case is listed in col. 2. Col. 3 gives the average Q_{alanine} value obtained with the media lacking the amino acid cited in col. 1; col. 4 gives the average Q_{alanine} value of the same media with addition of this amino acid. Col. 5 reports the average increase or decrease in Q_{alanine} resulting from addition of the amino acid (i.e. col. 4–col. 3); col. 6 gives the standard error of this difference, and col. 7 the value of P, the probability that an effect as great or greater than that recorded in col. 5 should arise by chance.

		Q_{alanine}				
Amino acid	Expts.	Without	With	Difference	S.E.	P
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Alanine	8	23·1	25·6	+2·5	4·8	0·6
Arginine	11	20·2	16·7	−3·5	4·0	0·4
Aspartic acid	8	21·9	19·9	−2·0	3·5	0·6
Cysteine	6	22·6	20·9	−2·6	4·5	0·6
Glycine	10	21·3	14·8	−7·5	2·6	**0·05**
Histidine	9	20·1	23·6	+3·5	2·8	0·4
Isoleucine	19	17·7	20·1	+2·4	2·5	0·3
Leucine	28	17·3	20·3	+3·0	1·5	**0·05**
Lysine	10	20·4	18·0	−2·4	2·6	0·4
Methionine	27	17·3	21·3	+4·0	1·6	**0·02**
Ornithine	6	22·0	16·0	−6·0	3·6	0·15
Phenylalanine	10	20·8	19·9	−0·9	4·2	—
Proline	10	23·0	20·0	−3·0	3·5	0·4
Serine	17	18·1	18·8	+0·7	3·2	—
Threonine	13	23·8	16·8	−7·0	3·0	**0·05**
Tryptophan	6	20·6	16·6	−4·0	3·3	0·4
Tyrosine	11	21·5	19·0	−2·5	3·1	0·4
Valine	13	22·5	19·3	−3·2	0·4	0·4

The entire series of results was treated in this way, and schedules similar to Table 2 were prepared for 18 different amino acids (excluding glutamic acid, which is present in the basal medium). The average stimulation or inhibition caused by each amino acid and the value of P are shown in Table 3. These results suggest that the high activity of complex media cannot be attributed to the action of one or a few specifically stimulatory amino acids. Most amino acids had relatively little effect on enzyme production when their average performance in a number of different combinations was assessed. Only leucine and methionine produced stimulation statistically significant at the level of $P = 0{\cdot}05$, and the average increase was less than 25 % in both cases. Even when leucine and methionine were both added to each of 20 different combinations of amino acids the average Q_{alanine} value increased only from 15·2 to 20·1 ($P=0{\cdot}05$). Depression of activity (significant at $P=0{\cdot}05$) was shown by glycine and threonine.

The effect of threonine is particularly interesting in view of its importance in stimulating enzyme activity in complex media. Sewell & King (1955) showed that when isoleucine and threonine were both omitted from a complete amino acid mixture, Q_{alanine} fell to the value of the basal medium although omission of either amino acid separately did not cause any great decrease in activity. We have confirmed this finding. These two amino acids thus appear to play a crucial role in complex media, but in the simpler systems considered in Table 3, isoleucine showed slight stimulation whilst threonine inhibited enzyme formation. A noteworthy feature of the results shown in Table 3 is that the majority of the amino acids listed appear to inhibit enzyme production although an acceptable level of statistical significance is approached only with threonine and glycine. The high activity obtained with casein hydrolysate and the complete amino acid mixture indicate the importance of amino acids in stimulating enzyme activity and Table 1 shows that the majority of amino acids are stimulatory when added singly to the basal medium. But under the conditions of Table 3, with small groups of amino acids, it seems that interactions unfavourable to enzyme formation must occur.

It is well known that correct balance of amino acids may sometimes be required for normal growth. Gladstone (1939) found that growth of *Bacillus anthracis* was inhibited by either valine, leucine, or isoleucine; but growth proceeded normally if all three were present in suitable proportions. He concluded that competition was taking place between these structurally-related compounds for possession of an enzyme centre necessary for the metabolism of all three; or perhaps their entry into the cell through a specific permease mechanism is involved (Cohen & Rickenberg, 1956). There were suggestions of such a relationship in our case: valine and isoleucine together inhibited growth less severely than did either alone. More interesting, however, were the effects of threonine and isoleucine, especially in view of the part they play in stimulating enzyme formation in complex media (Sewell & King, 1955; see above). When added singly to the basal medium both were growth inhibitors and were still more inhibitory when added together. Isoleucine was the only amino acid (of 17 examined) which enhanced the growth inhibition caused by threonine; conversely, the inhibition caused by isoleucine was much intensified by serine and threonine. Of 16 amino acids examined, only valine and leucine effectively annulled the combined threonine + isoleucine inhibition. The amino acids were, in all cases, added to the basal media at the level of 0·02 % of L-isomer.

The overall effect of different amino acids as stimulants or inhibitors of growth is shown in Table 4, constructed in the same way as Table 3. Comparison was made between pairs of media; one of each pair contained a particular group of amino acids and the other contained the same group plus the amino acid under consideration. Only leucine has an unambiguous action; it inhibits growth significantly. Nor can any correlation be established between action on growth and on enzyme formation. This is important since it goes far towards excluding the possibility that the results in Table 3 arise from 'age of culture' effects due to the varying action of the amino acids on the growth of the organism.

Table 4. *Effect of amino acids on growth*

Columns have same significance as in Table 3, except that cols. 3–6 refer to growth obtained after 36 hr. at 37°.

Amino acid (1)	Expts. (2)	Growth (mg./ml. × 10^2) Without (3)	With (4)	Difference (5)	S.E. (6)	*P* (7)
Alanine	7	20	17	− 3	4·5	0·6
Arginine	10	19	19	0	5	—
Aspartic acid	7	21	14	− 7	3	**0·05**
Cysteine	5	24	17	− 7	3	0·08
Glycine	9	21	20	− 1	5	—
Histidine	9	31	21	−10	7	0·3
Isoleucine	19	19	24	+ 5	3	0·15
Leucine	25	50	27	−23	6·5	**0·001**
Lysine	9	17	23	+ 6	6	0·3
Methionine	23	33	28	− 5	8·5	0·6
Ornithine	5	20	16	− 4	3·5	0·3
Phenylalanine	9	20	16	− 4	2·5	0·2
Proline	9	19	15	− 4	2·5	0·2
Serine	16	17	17	0	2	—
Threonine	13	20	23	+ 3	4	0·4
Tryptophan	5	23	28	+ 5	5·5	0·4
Tyrosine	10	21	21	0	4	—
Valine	12	18	17	− 1	3·5	—

Influence of purine and pyrimidine bases and essential metabolites

Although a complete mixture of amino acids produced suspensions of organisms comparable in enzyme activity with those obtained on nutrient broth or casein hydrolysate, we considered the possibility that amino acids might not be the only factors controlling enzyme production. In some cases enzyme formation will proceed only when the purine and pyrimidine constituents of the nucleic acid template are provided (Gale & Folkes, 1955). Shortage of any essential metabolite may disturb normal metabolism, and some compounds (e.g. pyridoxal and folic acid) are known to be specially concerned in the processes which lead to protein synthesis. Addition (mg/100 ml. basal medium) of nicotinic acid (0·5), calcium pantothenate (0·1), riboflavin (0·1), pyridoxal hydrochloride (0·05), folic acid (0·005) and biotin (0·001) did not enhance enzyme activity appreciably. Neither did the addition of yeast extract (0·1 %, w/v) appreciably increase the activity of casein hydrolysate medium. Guanine inhibited growth when added to the basal medium (Table 5), but had no influence on enzyme formation. Adenine, cytosine and uracil individually stimulated growth and, to a lesser extent, enzyme formation; together they were unable, however, to annul the inhibition caused by guanine. But when guanine was added to the basal medium along with either leucine (0·02 %, w/v) or methionine (0·02 %, w/v) it stimulated growth but retarded enzyme formation.

Table 5. *Effect of purines and pyrimidines on growth and ezyme formation*

Organisms were harvested after growth on the basal medium plus the indicated additions for 36 hr. at 37°. The growth (culture density at harvesting) and the enzyme activity of the harvested cells are recorded.

Additions to basal medium (0·02 % of each)							
Adenine	Guanine	Cytosine	Uracil	Leucine	Methionine	Growth (mg./ml. × 10^2)	Q_{alanine}
−	−	−	−	−	−	43	8
+	−	−	−	−	−	103	12
−	+	−	−	−	−	15	7
−	−	+	−	−	−	103	11
−	−	−	+	−	−	113	11
+	+	+	+	−	−	20	12
−	−	−	−	+	−	27	24
−	+	−	−	+	−	80	15
−	−	−	−	−	+	33	22
−	+	−	−	−	+	80	13

DISCUSSION

Specific amino acid requirements for the production of enzyme systems have been investigated in only a few cases. Gale (1940) reported that a strain of *Streptococcus faecalis* grew on a relatively simple medium but that its tyrosine decarboxylase activity was low unless casein hydrolysate was added to the medium; gelatin hydrolysate was less effective. *Escherichia coli* forms a number of enzymes only on relatively complex media. Optimal nitratase activity is obtained with casein hydrolysate; moderate activity was obtained on a medium containing six amino acids but the requirements were not determined precisely (Pollock & Wainwright, 1948; Wainwright, 1950). Hydrogenase formation was stimulated by a mixture of 17 amino acids but only to 60 % of the level attained with casein hydrolysate. Individually, glutamic acid, methionine, tyrosine, cysteine, serine, arginine and alanine (in descending order) showed some stimulation of enzyme formation; these seven amino acids together gave activity one-third of that obtained on casein hydrolysate (Billen & Lichstein, 1950). With formic hydrogenlyase, glutamic acid in high concentration gave as good enzyme activity as the complex media; certain acids which had no stimulatory action *per se* enhanced enzyme formation when simultaneously incorporated into the medium (Billen & Lichstein, 1951). When, however, Pinsky & Stokes (1952) re-investigated formic hydrogenlyase formation in *E. coli* under conditions which did not permit of cell division they obtained somewhat different results. A mixture of arginine + aspartic acid + glutamic acid gave 62 % of activity obtained on casein hydrolysate; and this figure was further increased by addition of cysteine, glycine, serine and threonine. They also listed six amino acids which diminished enzyme activity when added to the aspartic acid + glutamic acid + arginine mixture; and among these inhibitors was methionine, which was found to stimulate enzyme formation in Billen & Lichstein's (1951) experiments with growing organisms.

The present work thus falls into line with previous experience in suggesting that the effect of amino acids on enzyme formation is complex and the performance of any particular amino acid under given circumstances cannot be predicted easily. Thus there is nothing in the behaviour of threonine and isoleucine in the simpler amino acid mixtures to suggest that their simultaneous omission from the complete mixture would lead to drastic loss in activity. The development of a constitutive enzyme must depend on the presence of the appropriate genetic factors and an adequate supply of the requisite amino acids to the enzyme-synthesizing centre. Production of the enzyme will be restricted if the supply of any amino acid is limited either by the deficiency in the medium, a restricted degree of biosynthesis, blocks in the mechanism of transmission, or diversion to other metabolic pathways. Addition of an amino acid to a simple medium might accelerate enzyme production by providing a necessary component; on the other hand, it might delay it by competing for an enzyme handling a number of related amino acids (cf. Cohen & Rickenberg, 1956); or by creating an amino acid shortage through stimulation of the synthesis of other proteins. Results such as those reported and discussed here will be difficult to interpret as long as the complex interplay of these factors remains undetermined.

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REFERENCES

Billen, D. & Lichstein, H. C. (1950). Nutritional requirements for hydrogenase production in *Escherichia coli*. *J. Bact.* **60**, 311.

Billen, D. & Lichstein, H. D. (1951). Nutritional requirements for the production of formic hydrogenlyase, formic dehydrogenase, and hydrogenase in *Escherichia coli*. *J. Bact.* **61**, 515.

Cohen, G. N. & Rickenberg, H. V. (1956). Concentration specifique reversible des amino acides chez *Escherichia coli*. *Ann Inst. Pasteur*, **91**, 693.

Fairhurst, A. S., King, H. K. & Sewell, C. E. (1956). Studies in amino acid biogenesis; the synthesis of alanine from pyruvate and ammonia. *J. gen. Microbiol.* **15**, 106.

Gale, E. F. (1940). The production of amines by bacteria. 2. The production of tyramine by *Streptococcus faecalis*. *Biochem. J.* **34**, 846.

Gale, E. F. & Folkes, J. P. (1955). The assimilation of amino acids by bacteria. 21. The effect of nucleic acids in the development of certain enzymic activities in disrupted staphylococcal cells. *Biochem. J.* **59**, 675.

Gladstone, G. P. (1939). Inter-relationships between amino acids in the nutrition of *B. anthracis*. *Brit. J. exp. Path.* **20**, 189.

Gordon, W. G., Semmett, W. F., Cable, R. S. & Morris, M. (1949). The amino-acid composition of α-casein and β-casein. *J. Amer. chem. Soc.* **71**, 3293.

Pinsky, M. J. & Stokes, J. L. (1952). Requirements for formic hydrogenlyase adaptation in nonproliferating suspensions of *Escherichia coli*. *J. Bact.* **64**, 151.

Pollock, M. R. & Wainwright, S. D. (1948). Relationship between nitrase and tetrathionase adaptation and cell growth. *Brit. J. exp. Path.* **29**, 223.

Sewell, C. E. (1954). *Amination of Pyruvic acid in Micro-organisms*. Thesis: University of Liverpool.

SEWELL, C. E. & KING, H. K. (1945). A threonine-isoleucine relationship in *Bacillus subtilis*. *Biochem. J.* **60**, xxi.

SHAH, P. C., KING, H. K., HOLLIS, B. & FAIRHURST, A. S. (1957). Studies in amino-acid biogenesis: optical activity of amino acids formed by reductive amination in *Bacillus subtilis*. *J. gen. Microbiol.* **17**, 620.

WAINWRIGHT, S. D. (1950). Formation of a bacterial adaptive enzyme system in absence of substrate: production of nitratase by *B. coli* without nitrate. *Brit. J. exp. Path.* **31**, 495.

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Bisset, K. A. (1959). *J. gen. Microbiol.* **20**, 89–90

Some Characters of *Rhizobium* Strains from Tropical Legumes

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SUMMARY: Twelve strains of *Rhizobium* sp. from wild and cultivated leguminous plants growing in the Belgian Congo were examined. All were bacillary or filamentous, weakly Gram-positive, and produced occasional refractile, heat-resistant spores; all grew alternatively in the form of very tiny, Gram-negative rods with polar flagella (if motile), and without spores. Botanical evidence suggests that the host plants are primitive members of the family Leguminosae, and if the characters of these strains of *Rhizobium* are also to be regarded as primitive, this evidence supports the view that *Rhizobium* is related to *Azotobacter* and the Bacillaceae.

It was suggested (Bisset, 1952) that *Rhizobium* is an aberrant genus of Bacillaceae, related to *Bacillus polymyxa*, and further (Bisset, 1955), that the genus *Azotobacter* and the nitrogen-fixing clostridia are all members of the same relatively close group. Support for this theory has recently been provided by Hino & Wilson (1958), who have shown that nitrogen is fixed by bacilli which closely resemble *Bacillus polymyxa*. In an analytical review, Norris (1956) brought evidence that the tropical genera of Leguminosae, the host plants of the *Rhizobium* symbionts, are relatively primitive, and that their strains of *Rhizobium* (the so-called cow-pea type) may also be primitive. It is thus of interest to examine the characters of *Rhizobium* from tropical legumes, in order to discover whether these are especially suggestive of a relationship with the *Bacillus polymyxa* group; and this proves to be the case.

METHODS

Twelve isolates of *Rhizobium* from tropical legumes in the Belgian Congo were obtained by the kindness of Dr C. Bonnier from the collection maintained in the Institut Agronomique, Gembloux, for experimental purposes, including cross-inoculation studies; the host plants were in the genera *Arachis*, *Desmodium*, *Stylosanthes*, *Pueraria*, *Vigna*, *Canaralis*, *Mimosa*, *Calopogonium*, *Afrormosia*, *Crotalaria*, *Pterocarpus* and *Deweraea*, of which the first six are cultivated plants, and the remainder wild. The bacteria were cultivated on cooked potato slopes, upon which they grew luxuriantly at 20°.

OBSERVATIONS

As with most strains of the genera *Rhizobium* and *Azotobacter*, these bacteria grew unpredictably in more than one morphological phase. In this case they were all very similar and adopted two forms. When repeatedly subcultured they grew as large, rather irregular, weakly Gram-positive bacilli and filaments, up to $1{\cdot}5\,\mu$ in diameter, with very occasional refractile spores. When subcultured at intervals of a week or longer they grew mainly as exceedingly tiny, Gram-negative rods, *c.* $0{\cdot}3\,\mu \times 1\,\mu$. Under the electron microscope these could be observed to have polar flagella, usually two in number, but not in some isolates, e.g. that from *Mimosa*. These minute bacilli resembled the gonidia of other *Rhizobium* spp. (Bisset, 1952) rather than the bacterium-like growth form typical of isolates from cultivated temperate-zone plants; but they resembled the latter in that they were capable of reproducing indefinitely in this phase. The bacterium-like forms were seen, but only very occasionally.

Five isolates were tested for heat resistance. In the sporogenous phase they survived 20–30 min. at 80°. In the minute phase they failed to survive 50° for more than a few minutes.

Biochemical reactions were, as with other strains of *Rhizobium*, very variable. Six either failed to grow in peptone water sugars or to ferment any sugar; the remainder fermented glucose, mannitol and sucrose, but never dulcitol; four produced acid, and two, acid and gas after 14 days. Culturally they resembled other *Rhizobium*, with pale mucoid colonies.

DISCUSSION

The twelve isolates of *Rhizobium* from supposedly primitive tropical legumes were morphologically much alike and showed characters suggestive of phylogenetic primitiveness, on the theory that the ancestral form resembled *Bacillus polymyxa*. All were capable of adopting a weakly Gram-positive bacillary or filamentous form, and produced spores. Some produced acid and gas from carbohydrates. Similar characters are found, but less regularly, in European *Rhizobium* (Bisset, 1952), and these adopt very freely a bacterium-like form which in the tropical strains is much less common. Both produce tiny, polar-flagellate swarmers, as do numerous other types of bacteria, notably *Azotobacter*. It is worthy of emphasis that these tropical *Rhizobium*, although showing very obvious and regular signs of their relationship with the sporing bacilli, are nevertheless entirely distinct from any common species of *Bacillus*. By comparison with temperate-zone strains they are much less variable in their morphology, although still much more so than saprophytic bacilli.

REFERENCES

Bisset, K. A. (1952). Complete and reduced life-cycles in *Rhizobium*. *J. gen. Microbiol.* 7, 233.

Bisset, K. A. (1955). Evidence from the cytology of *Azotobacter chroococcum* of a relationship with *Rhizobium* and the Bacillaceae. *J. gen. Microbiol.* 13, 443.

Hino, S. & Wilson, P. W. (1958). Nitrogen fixation by a facultative bacillus. *J. bact.* 75, 403.

Norris, D. O. (1956). Legumes and the *Rhizobium* symbiosis. *Emp. J. exp. Agric.* 24, 247.

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Dorey, M. J. (1959). *J. gen. Microbiol.* **20**, 91–104

Some Properties of a Pectolytic Soil Flavobacterium

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SUMMARY: A pectolytic *Flavobacterium* sp. isolated from soil is described. It possesses a typical bacterial polygalacturonase but no pectin methyl esterase.

Most previous studies on micro-organisms which decompose the pectic substances of plants have centred round three topics: flax-retting, fruit-juice canning and the soft-rot diseases of plants. The only reported observation on the non-pathogenic pectolytic microflora of soil were made by Wieringa (1949). Wieringa devised a pectate gel plate which used the ability of sodium pectate to gel at neutral pH values in the presence of calcium ion, thus producing a firm surface which could be streaked with a bacterial culture. On plating dilutions of an unspecified soil on this selective medium Wieringa found large numbers of actinomycetes. It has been assumed, largely from aetiological studies of the organism, that *Erwinia carotovora*, which causes a soft-rot of plant storage organs, occurs in a wide range of soils; but the support for this theory seems largely to be the report (Kerr, 1953) that 20 soil samples from the North of Scotland all contained this organism. The present paper describes the isolation from soil of a hitherto undescribed organism which is pectolytic, possesses a polygalacturonase but no pectin methyl esterase, and is nutritionally non-exacting. These properties made it a suitable tool for more detailed studies on the production and properties of the polygalacturonase.

METHODS

Soil samples were collected aseptically in sterile containers and were tested with the minimum of delay. The pectate gel medium was prepared after the method described by Richards & Fouad (1954) except that 2% (w/v) sodium pectate was used for pouring plates for streaking. The soil percolator used was after a model devised by Dr G. Metcalfe (private communication), similar in essentials to that of Lees (1949), but with the soil column and the reservoir. plugged with non-absorbent cotton wool, thus making the apparatus simpler and more adjustable. The maintenance of aseptic conditions was aided by using, to circulate the fluid, positive air pressure from a small pump, instead of suction.

Pectin (Brown Ribbon brand) and sodium pectate (Red Ribbon brand) were obtained from Unipectin Ltd., Zurich, and were purified in the manner described by Wood (1955). The methoxyl content of these substances was estimated by Zinn's (1955) modification of the Zeisel technique.

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Viscosity measurements were made with a No. 2 Viscometer (British Standards No. 188) in a water bath at 25°; the time taken for water to run through was 19–20 sec. and for pectin solutions between 28 sec. and 3 min.

Assay of polygalacturonase. A plate assay devised for fungal polygalacturonase by Dingle, Reid & Solomons (1953) was adapted for assaying the bacterial enzyme. Sodium pectate (1 %, w/v) in 0·2 M-tris (2-amino-2-hydroxymethylpropane-1:3-diol) + hydrochloric acid buffer (pH 8·0), with 0·01 % (w/v) salicylanilide to inhibit micro-organisms, was solidified with 1·5 % (w/v) water-washed agar; plates (9 cm. diam.) were poured with 10 ml. of this medium. The material to be tested was taken up in 'fish spines' (capacity 0·04 ml.) and 12 spines used on each plate. The concentration of calcium in the medium was such that the addition of more calcium to test material only depressed enzyme activity. The plates were incubated for 18 hr. at 37° and developed by flooding with 5 N-HCl. Clear circular zones where the sodium pectate had been hydrolysed were revealed round the spines, surrounded by a denser ring on a faintly opaque ground. Washing of the agar and purification of the sodium pectate markedly improved the sharpness of definition of the inner zone, the diameter of which was measured. With this test a distinct zone of activity was detected with culture fluid diluted to 1/512 in tris buffer (= 0·00008 ml. culture fluid). Arbitrary 'spine-plate units' of polygalacturonase activity were calculated by squaring the radius (mm.) of the clear zone, and determining graphically the quantity of enzyme present relative to the amount which would give a zone radius of 5 mm. (= 1 unit). A further test plate in which the calcium concentration was controlled was devised by using silica gel in place of agar. A layer of silica sol (Smith, 1951) containing the desired amount of calcium as $CaCl_2$, with NaCl added when necessary to assist gelling, was poured into a Petri dish; when gelled, a further layer of sol containing sodium pectate (final concentration 0·5%, w/v) and tris buffer (pH 8) was poured on top. The culture filtrate tested was freed from Ca^{++} by passage through a mixed ion-exchange resin column containing IRC 50 and IR 4 B (Rohm and Haas, Philadelphia, U.S.A.); the enzyme content was unaffected by this treatment. For permanent preparations of agar test plates the material was floated out of the dish, captured on a glass lantern slide and allowed to dry at room temperature (Rondle & Carman, 1956). The slides were stained for 10 min. with 1 % (w/v) safranin, washed until the stain remained only in the background, dried and stored.

Bacterial culture filtrates were tested for the presence of pectin methyl esterase by a qualitative method devised by Smith (1958) which relies on the detection of a decrease in pH value of the enzyme + substrate mixture because of the release of acidic groups.

Organisms. For use in comparative determinative tests, cultures of *Flavobacterium* spp. were obtained from various sources. The National Collection of Industrial Bacterial (NCIB) supplied 9 strains: no. 8535 (*F. aquatile*); 8185 (*F. arborescens*); 8204 (*F. aurantiacum*); 8205 (*F. denitrificans*); 8207 (*F. sulphureum*); 8615 (*Pseudomonas putrificans*, an organism which was classified in the past as *Flavobacterium*); 8188 (*F. suaveolens*) 8186 (*F. esteroaro-*

maticum); 8187 (*F. flavescens*). Dr E. Windle Taylor (Metropolitan Water Board, London) supplied a culture of *F. aquatile* (NCTC 9758), which Weeks (1955) pronounced to be identical with the original published description, in contrast to all other strains so named. A *Flavobacterium* sp. which produced L forms was obtained from Dr L. Dienes (Massachusetts General Hospital, Boston, Mass., U.S.A.), but unfortunately the culture as received no longer appeared to possess this property. A *Flavobacterium* sp. which degraded α-conidendrin was obtained from Dr W. A. Konetzka (Indiana University, Bloomington, Indiana, U.S.A.), who also supplied a sample of α-conidendrin. *F. resinovorum* which degraded rosin was obtained from Dr P. Daste (University of Paris). Mr W. K. Smith and Dr C. B. Taylor (Imperial Chemical Industries Ltd., Akers Laboratory, Welwyn, Hertfordshire) provided several cultures of *Erwinia carotovora* and *Xanthomonas campestris*. A culture labelled *F. aquatile*, isolated by the late Dr T. Richards from a water sample, was also tested.

RESULTS

Thirty-one samples were collected in the Thames Valley and Cotswolds from relatively undisturbed sites (woodland and pasture, but not arable land). Samples were percolated in the Metcalfe modification of the apparatus of Lees with 1 % pectin solutions and the flora in the reservoir fluid was examined microscopically. After 4 days of percolation at room temperature a mixed flora developed and the pectin was attacked; the soil column was then washed for 6 hr. with sterile distilled water, and fresh pectin solution percolated. In 18 hr. large numbers of non-motile Gram-negative rods ($4–5 \times 0{\cdot}5\,\mu$) appeared, and samples of the microscopically pure percolate were streaked on pectate gel.

Later it was found more convenient to scatter soil crumbs directly on plates of pectate gel medium. In this case regions of liquefaction developed around the crumbs after 3 days at 25°; microscopically a mixed flora was observed in which a long Gram-negative rod predominated in the early stages. This mixed culture was re-streaked on pectate gel medium. Material from isolated regions of liquefaction (=isolated colonies of the pectolytic rod) was re-streaked on pectate gel medium, and finally on yeast extract agar. Subcultures of single colonies from these latter plates behaved as pure cultures. In every case the organism isolated was a pectolytic non-motile Gram-negative non-sporing catalase-positive rod, possessing a yellow pigment, producing neither acid nor gas in sugar media containing peptone, but producing acid from some sugars in defined medium without peptone. It grew well on ordinary laboratory media, and produced no change in litmus milk. The organism was therefore regarded as a flavobacterium.

In one isolation experiment dilutions of a soil sample were plated on pectate gel medium, and a large number of actinomycete colonies and a few fungi were observed on these plates. No attempt was made to isolate pectolytic anaerobes. The aerobic pectin degraders *Bacillus polymyxa* and *Erwinia carotovora* did not appear on the plates, although stock cultures of these organisms grew well on the pectate gel medium. The addition of bile salts to the pectate gel medium suppressed the flavobacterium and resulted in the isolation of a pseudomonad; *E. carotovora* was not found.

Twenty-five isolates of what appeared to be pectolytic flavobacteria, derived from 13 soil samples, were studied in more detail. In addition, selected cultures of these organisms were compared with a number of strains of named flavobacteria obtained from culture collections and from individual workers. Examination showed that the organisms listed as *Flavobacterium* spp. formed a heterogeneous group. Some organisms were Gram-positive (most only transiently so) and others were Gram-negative. There seems no reason for including Gram-positive organisms in the genus *Flavobacterium* when these can all be included in the genus *Corynebacterium*. *Flavobacterium sulphureum* NCIB 8207 was manifestly no longer like the organism originally described; as received it was identified as a strain of *Bacillus circulans*. *Pseudomonas putrificans* NCIB 8615 was included in the study since it has at times been classified as a *Flavobacterium* sp.; this was one of the organisms which were Gram-positive in very young cultures.

In view of the work of Weeks (1955) Dr Windle Taylor's culture of *Flavobacterium aquatile* was accepted as a quasi type species of the genus. Some of the more significant results obtained are recorded in Table 1. The eight strains from soil (69, 72, 76, 81, 85, 87, 91, 94) formed a homogeneous group which differed significantly from other *Flavobacterium* spp., but were sufficiently similar, especially in pigment production, to be regarded as members of that genus. They are, therefore, designated a new species, with the specific epithet *pectinovorum*, derived from *pectinus*—pectin and *vorare*—to swallow.

Flavobacterium pectinovorum n.sp.

All observations at 25° unless otherwise stated.

Gram-negative, non-motile, non-capsulated, non-sporing rods $3 \times 0{\cdot}5\,\mu$ in young cultures in nutrient broth and pectin-containing media, outline regular, end walls rounded, becoming irregular with forms up to $15\,\mu$ long in older cultures. Colonies on yeast extract agar at 2 days 0·5 mm. diameter, circular, convex, matt, pale yellow, margin lobed. On pectate gel medium colonies visible at 2 days as depressions in the medium, which later liquefied; odour sweet, rich, fruity. Good growth on cooked potato.

Yellow pigment, produced on all media, behaved as a carotenoid alcohol, moving as a single band on a chalk column; absorption peaks in carbon disulphide at 404, 434 and 462 mμ.

Indole, methyl red and Voges-Proskauer tests negative; no growth in Koser's citrate. Ammonia not produced in peptone water or arginine broth; H_2S not produced from peptone water + cysteine. Nitrate reduced to nitrite but not to nitrogen. Gelatin stabs incubated at room temperature: crateriform liquefaction in 5 days, stratiform in 2 weeks. Frazier's gelatin hydrolysed, tributyrin hydrolysed; pectate gel liquefied, horse blood not lysed, methylene blue not reduced. Catalase positive, though rather weak. Litmus

		F. aquatile 1	*F. pectinovorum* isolates								*F. resinovorum*	*F.* sp. (α-conidendrin)	*F.* sp (L forms)	*F. aquatile* 2	*F. aquatile* 3	*F. arborescens*	*F. aurantiacum*	*F. denitrificans*	*F. sulphureum*	*Ps. putrificans*	*F. suaveolens*	*F. esteroaromaticum*	*F. flavescens*
			94	91	87	85	81	76	72	69													
Gram reaction 4 hr.		−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+	+	+
Gram reaction 18 hr.		−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	−
Motility		−	−	−	−	−	−	−	−	−	±	+	−	−	+	+	−	+	+	+	+	+	+
Grow at 37°		−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Survive 45°/10 min.		−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	+
Survive 40°/10 min.		−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grow in glucose + salts		−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Grow in peptone water	+3 % NaCl	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+	+	+	+
	+1 % NaCl	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduced	to nitrite	−	+	+	+	+	+	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−	−
	to nitrogen	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Acid from carbohydrates without peptone	glucose	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−
	maltose	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	+	+	+	+
	xylose	−	+	+	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	−	−	−	−
	arabinose	−	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Acid from carbohydrates + peptone	glucose	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	+	+	+	+	+
	maltose	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	−	+	+
	glycerin	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+	+	+
	xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Indole production		−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−
Methyl red reaction		−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Voges-Proskauer reaction		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth on citrate		−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−
Anaerobic growth		+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−
Methylene blue reduction		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Gelatin stab liquefied		+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	−	+	+	+	−	−
Ammonia from urea		−	−	−	−	−	±	−	−	−	+	±	−	−	−	−	+	+	−	−	−	−	−
Ammonia from arginine broth		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Grow in urea as N source		−	+	+	+	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
H_2S from cysteine		+	−	−	−	−	−	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Haemolysis		−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+	+	+	+
Tributyrin hydrolysed		−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	±	±	±	±

Flavobacterium aquatile strains: 1 = Dr Windle Taylor's strain NCTC 9758; 2 = Dr T. Richard's strain; 3 = NCIB 8535.
Flavobacterium sp. (L forms) = Dr Dienes's organism, producing L forms.
Flavobacterium sp. (α-conidendrin) = α-conidendrin decomposer isolated by Dr Konetzka.

milk in 5 days clearing 1–2 mm. at top; in 10 days soft curd and litmus partially reduced, reaction neutral; then shifting slowly acid in 1–2 months.

Trace of acid from carbohydrates added to peptone media; in media containing only carbohydrate and inorganic salts, acid from glucose and maltose (5 days), sucrose, lactose, xylose, arabinose and inulin (12 days), no growth in salicin or glycerol. A few strains grew and produced acid from salicin and glycerol; one strain did not grow with xylose or arabinose as sole carbon and energy source.

Optimum temperature 25°, good growth at 30°, none at 37°; thermal death point 45°/10 min. Growth in peptone water containing NaCl 0·1 to 1 % (w/v), not in 3 %. Urea poorly utilized as a N source, if at all; NH_4^+ utilized: ammonia not liberated from Christensen's urea medium. No growth anaerobically.

Distinctive characters: able to grow on medium containing pectic substances as sole carbon source. Possesses an inducible polygalacturonase.

Stock cultures maintained on yeast extract and glucose yeast extract agar died, often within a week of inoculation; but cultures maintained in media including pectic materials remained viable at room temperature for more than a year. When large inocula were seeded on yeast extract agar plates only a few colonies developed, suggesting that the medium contained inhibitory material. The addition of laked red cells from 0·002 ml. horse blood/ml. medium was sufficient to relieve this inhibition, as were purified horse liver catalase at a final concentration of 0·03 mg./ml. and MnO_2; it was therefore concluded that the inhibitor present in the nutrient agar was hydrogen peroxide (Proom, Woiwod, Barnes & Orbell, 1950).

Strain no. 81 was designated as the type and deposited with the National Collection of Industrial Bacteria.

Production of polygalacturonase

Experiments were performed to determine the conditions under which *Flavobacterium pectinovorum* and *Erwinia carotovora* produced maximum quantities of polygalacturonase. Organisms were grown aerobically in 25 ml. medium in 100 ml. flasks shaken at 100 strokes/min. (2 cm. throw) in a water bath at 25°. The inoculum was 0·1ml. of a 25 hr. culture in yeast-extract peptone medium. *F. pectinovorum* isolate 81 was the strain used throughout; in many experiments *E. carotovora* (Dr Taylor's strain, numbered 16F here) was compared with it, and in a few cases other strains of *E. carotovora* were used. The medium contained (%, w/v): $NH_4H_2PO_4$, 0·2; $MgSO_4 . 7H_2O$, 0·02; $MnSO_4 . 4H_2O$, 0·002; $FeCl_3$, 0·0005; $CaCl_2$, 10^{-3}M (=0·001 %) except where otherwise stated; bromthymol blue 1·6 % (w/v) ethanolic solution, 0·1 ml. To this basal solution were added different amounts of peptone or yeast extract, and 1 % (w/v) of carbohydrate (glucose, galacturonic acid, pectin, sodium pectate). The carbohydrate and phosphate solutions were autoclaved separately and added aseptically to the solution of salts with or without peptone and yeast extract. The pH value of cultures was noted daily, and where necessary adjusted with sterile N-NaOH or HCl. It was difficult to adjust aseptically the pH value of 25 ml.

quantities of fluid, and in some cases it was impossible to maintain the pH value within the desired range by this method. Where the pH value varied by more than 0·2 unit in spite of correction, this was recorded. The amount of polygalacturonase was measured daily by the spine plate technique. Experiments lasted 2 or 3 days; in nearly all cases maximum enzyme production was achieved on the second day and later experiments were therefore run only for 2 days. At the end of the experiment 10 ml. samples were centrifuged and the organisms resuspended in Ringer's solution. The amount of material present was estimated nephelometrically by means of a previously constructed calibration curve. In other experiments the number of living organisms was estimated daily by the Miles & Misra (1938) method. Some of the data obtained are recorded in Table 2.

Flavobacterium pectinovorum isolate 81, and *Erwinia carotovora* 16F were grown in media containing the inorganic salts noted above, together with 1% (w/v) of one of the following substances or mixtures: glucose, sodium pectate, peptone, peptone + glucose, peptone + sodium pectate; at five pH values: 7·8, 7·4, 7·0, 6·6 and 6·2. The results at pH 7·4 are recorded in lines 1 to 10, Table 2. With glucose as carbon source both cultures grew poorly without peptone; with added peptone *F. pectinovorum* isolate 81 produced no enzyme, but *E. carotovora* 16F produced up to 4·7 spine plate units. With peptone alone isolate 81 again produced no enzyme, but *E. carotovora* 16F produced up to 8·6 units. *E. carotovora* 16F did not grow on sodium pectate without added peptone, but isolate 81 grew well and produced up to 7·7 units. Here the pH value remained fairly stable and below pH 7·0 enzyme was produced very slowly and in small amounts, and not at all at a pH value 6·2. With added peptone isolate 81 only produced enzyme (up to 14 units) at the lower pH values, but *E. carotovora* 16F produced enzyme (up to 65 units) over the whole range. In all cases the pH value increased rapidly, and it seems likely that polygalacturonase produced by *F. pectinovorum* isolate 81 is more unstable above pH 8 than is the enzyme produced by *E. carotovora* 16F. The latter organism produced polygalacturonase at pH 6·2, but isolate 81 not until the pH value had risen (uncontrolled) above 7. The optimum pH value for enzyme production by both organisms when growing was 7·4–7·6.

Flavobacterium pectinovorum isolate 81 was grown in various media compounded as follows: sodium pectate 1% (w/v) + peptone at 0, 0·01, 0·1, 0·2, 0·5, or 1% (w/v); with or without yeast extract at 0·0003, 0·03, 0·06, 0·15 or 0·3% (w/v); inorganic salts as previously recorded; initial pH 7·0, not adjusted during growth. The optimum peptone concentration for polygalacturonase production was in the range 0·01–0·2%; the addition of yeast extract made only a slight difference; optimum enzyme concentration was obtained with 0·1% (w/v) peptone + 0·03% (w/v) yeast extract. The calcium concentration was varied in a further experiment with isolate 81 grown on a medium containing sodium pectate 1%, peptone 0·1%, yeast extract 0·03% (w/v) and inorganic salts, at pH 7·5. Good yields of enzyme were obtained throughout the range studied (nil to 10^{-2} M-calcium added).

The optimum temperature for enzyme production was determined with

Table 2. *Production of polygalacturonase by* Flavobacterium pectinovorum *isolate* 81 *and* Erwinia carotovora 16 F *in shake flask cultures under different conditions*

Organism	Peptone (%)	Yeast extract (%)	Carbon source (%)	pH value	Inoculum $\times 10^{-6}$ organisms	Polygalacturonase spine plate units		pH rose or fell to	Bacterial numbers $\times 10^{-7}$		Tem (°)
						(24 hr.)	(48 hr.)		(24 hr.)	(48 hr.)	
16 F	0	0	G	7·4	14	0	0	7·0	0·37	1·85	25
	0	0	N	7·4	1·1	0	0	—	<0·1	<0·1	—
	1	0	—	7·4	14	2·2	9·2	8·3	14	190	—
	1	0	G	7·4	14	0·1	4·3	7·7	67	910	—
	1	0	N	7·4	1·1	21·5	49	8·6	15	1400	—
81	0	0	G	7·4	80	0	0	6·8	2·3	19·5	—
	0	0	N	7·4	3·1	0	4·2	—	2·8	35	—
	1	0	—	7·4	80	0	0	8·2	18	650	—
	1	0	G	7·4	3·1	0	0	6·2	1·1	620	—
	1	0	N	7·4	3·1	0	0	8·2	75	320	—
	0·01	0	N	7·0	5·1	0	11·8	—	—	196	—
	0·01	0·003	N	7·0	5·1	0	9·1	—	—	140	—
	0·1	0	N	7·0	5·1	0	9·1	7·4	—	320	—
	0·1	0·03	N	7·0	5·1	0·8	23	7·6	—	330	—
	0·2	0	N	7·0	5·1	0	11·8	7·5	—	320	—
	0·2	0·06	N	7·0	5·1	0	9·9	7·7	—	320	—
	0·5	0	N	7·0	5·1	0	7·5	7·8	—	340	—
	0·5	0·15	N	7·0	5·1	0	7·5	7·9	—	380	—
	1	0	N	7·0	5·1	0	0·6	8·1	—	340	—
	1	0·3	N	7·0	5·1	0	0	8·2	—	480	—
16 F	1	0	N	7·6	1·4	15	11·6	8·4	—	225	35
	1	0	N	7·6	1·4	52	39	8·8	—	280	30
	1	0	N	7·6	1·4	39	52	8·6	—	320	25
	1	0	N	7·6	1·4	30	57	8·3	—	335	20
81	0	0	N	7·6	0·72	0	0	—	—	0	35
	0	0	N	7·6	0·72	0	0	—	—	0	30
	0	0	N	7·6	0·72	0	5·7	—	—	205	25
	0	0	N	7·6	0·72	0·6	1·6	—	—	113	20
	0·1	0·03	N	7·0	0·72	0	0	—	—	0	35
	0·1	0·03	N	7·0	0·72	0	0	7·5	—	87	30
	0·1	0·03	N	7·0	0·72	5·5	20	7·7	—	390	25
	0·1	0·03	N	7·0	0·72	3·7	8·9	7·5	—	335	20
16 F	1	0	P	7·5	1·5	35	82	8·5	—	340	25
	1	0	N	7·5	1·5	20	27	8·8	—	340	—
	0·1	0·03	P	7·0	1·4	19	27	—	—	250	—
	0·1	0·03	N	7·0	1·4	11	12	7·9	—	290	—
81	0·1	0·03	P	7·0	0·94	3·7	7	7·5	—	180	—
	0	0	Ga	7·5	0·75	1·7	0·6	—	—	80	—

G = glucose; N = sodium pectate; P = pectin; Ga = galacturonic acid. Where two estimates of bacterial nur bers are given these were made by the Miles & Misra technique, otherwise the 48 hr. count was ma nephelometrically.

Flavobacterium pectinovorum isolate 81 grown in a medium containing sodium pectate 1 % (w/v) as sole carbon source, at pH 7·5, and one containing sodium pectate 1 % (w/v) + 0·1 % peptone + 0·03 % yeast extract at pH 7·0; and with *Erwinia carotovora* 16 F grown on sodium pectate 1 % + 1 % peptone (pH 7·5). Growth at 35°, 30°, 25° and 20° was studied; all flasks were duplicated. *E. carotovora* 16 F produced enzyme at all temperatures, though much less at 35°;

isolate 81 died out at 35°, and only grew at 30° in the peptone-containing medium; enzyme was only produced at 25° and 20°.

The influence of the rate of shaking was studied with *Flavobacterium pectinovorum* isolate 81 in a medium containing sodium pectate 1 % (w/v) at pH 7·5 and on one containing sodium pectate 1 % + 1 % peptone at pH 6·2. Three different conditions of shaking were used: static and shaken at 60 or 90 strokes/min. (normally 100). Both growth and enzyme production were greatly enhanced at the higher rates. Pectin was compared with sodium pectate as an inducer of polygalacturonase formation when incorporated into medium containing peptone 0·1 % (w/v) and yeast extract 0·03 % (w/v). *Erwinia carotovora* 16F produced more enzyme in the pectin-containing medium, while *F. pectinovorum* isolate 81 produced more in the sodium pectate medium. Lastly, isolate 81 was inoculated into a medium containing Seitz-filtered 1 % (w/v) galacturonic acid as sole carbon source, with inorganic salts, at pH 7·5; it produced enzyme after 24 hr., but this disappeared after a further 24 hr. incubation.

Heavy crops of *Flavobacterium pectinovorum* isolate 81 were grown in media containing peptone or peptone + glucose; washed suspensions of these organisms were incubated and shaken in the presence of sodium pectate. These suspensions became adapted to produce polygalacturonase; the period needed for adaptation was 24–48 hr., longer than would be required to grow a crop of organisms from a small inoculum. In pectate-containing media isolate 81 preserved the normal rod shape even after a month of incubation, but when grown in yeast extract medium the organisms became long (up to 50μ) and irregular in shape and staining properties within 24 hr. The failure of these organisms to adapt quickly to produce polygalacturonase might be connected with the presence of these involution forms.

Studies of the polygalacturonase produced by Flavobacterium pectinovorum *isolate* 81

Culture filtrates of *Flavobacterium pectinovorum* isolate 81 were studied to determine the properties of the pectic enzyme present, and culture filtrates of *Erwinia carotovora* isolate 16F were compared with these. Samples of culture fluids from isolate 81 grown under a range of conditions were tested for the presence of pectin methyl esterase, and were all found negative. Three isolates of *E. carotovora* and one of *Xanthomonas campestris* all produced the esterase when grown on a pectin-containing medium. It was therefore concluded that *F. pectinovorum* isolate 81 produced no pectin methyl esterase, but only a hydrolytic polygalacturonase type of enzyme or enzymes.

The initial stages of breakdown of pectin by polygalacturonase were followed viscometrically. Ten ml. of 1·5 % (w/v) purified pectin solution was mixed with 5 ml. 0·2 M-tris buffer (pH 8·0), 2 ml. 0·1 M-$CaCl_2$ and 3 ml. diluted culture fluid. The initial relative viscosity was *c.* 5 units, decreasing to 1·5 in 30 min. with active preparations. Enzyme preparations were diluted until it was possible to make an accurate measurement of the initial rate of viscosity decrease, and at high dilutions this rate was proportional to the enzyme concentration (Fig. 1). Using this graphic method an estimate of the relative strength of two different enzyme preparations was obtained (Fig. 2).

Twofold dilutions in tris buffer of culture filtrates of *Erwinia carotovora* 16F and *Flavobacterium pectinovorum* isolate 81 were tested by the spine plate method. Each dilution up to 1/256 was tested on two plates with 12 fish-spines/plate. The mean diameters of the clear zones were measured. When the square of the radius of the clear zone was plotted against the log of enzyme concentration, the data for isolate 81 fell on a straight line (fig. 3). The lowest dilution produced a readable response, and the controls (autoclaved enzyme) no response at all. The data for *E. carotovora* 16F, however, presented a more complex picture; in particular the angle of slope became greater at higher enzyme concentrations, so giving a false impression of high potency. The two samples of culture filtrate from isolates 16F and 81, respectively, which had been carefully assessed by the viscometric method, were tested by serial dilution on fish-spine plates, after the 16F sample, which was the stronger, had first been diluted to a potency equivalent to the sample of isolate 81 filtrate. The results are graphed in Fig. 4. Isolate 81 filtrate again gave a straight line while *E. carotovora* 16F filtrate showed a distinct curve; but the two solutions gave almost the same readings where the diameter of the clear zone was *c.* 10 mm. Graphs of this type were used to derive the spine plate units (SPU) of enzyme present in test solutions, assuming that a solution of potency 1 unit produces a 10 mm. diameter clear zone. The width of the opaque annulus surrounding the clear zone on the spine plate varied considerably; it was not directly correlated with the presence of pectin methyl esterase in the test

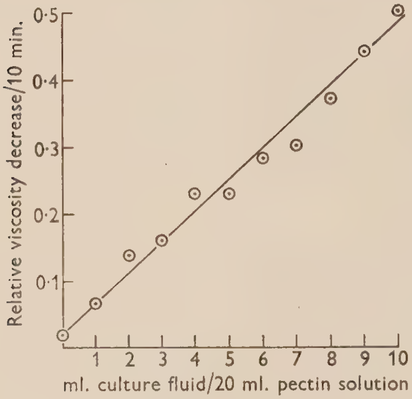

Fig. 1

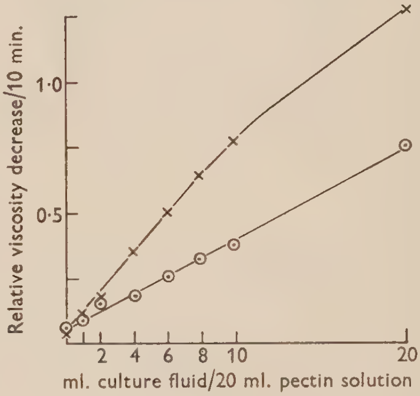

Fig. 2

Fig. 1. **Initial rate of pectin breakdown (followed viscometrically) plotted against concentration of *Flavobacterium pectinovorum* polygalacturonase.**

Fig. 2. As Fig. 1. *Erwinia carotovora* polygalacturonase, × ; *Flavobacterium pectinovorum* polygalacturonase ⊙.

Flavobacterium pectinovorum was grown in a medium containing sodium pectate 1% (w/v), 0·1 % peptone and 0·03% yeast extract; *Erwinia carotovora* in 1 % (w/v) sodium pectate and 1 % peptone.

solution. At higher potencies the spine-plate test is less accurate, and dilution down to a range of 1–10 SPU is recommended.

The hypoiodite method for the determination of release of reducing groups (Jansen & MacDonnell, 1945; Kraght & Starr, 1953) was used as a check on the other two methods. The method followed was the same as Kraght & Starr's, but with the sodium pectate dissolved in tris buffer (pH 8). Culture fluids of *Erwinia carotovora* 16F liberated 0·03 m-equiv. reducing groups/30 min,/2·5 ml. culture filtrate, flavobacterium isolate 81 0·02 m-equiv. These figures are near those quoted by Kraght & Starr.

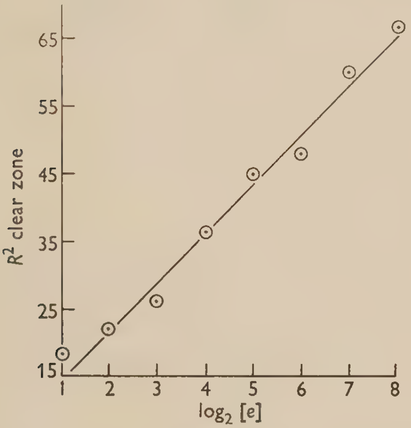

Fig. 3

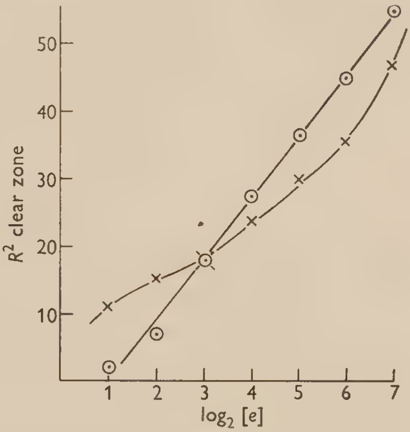

Fig. 4

Fig. 3. Area of clear zone on spine plate plotted against serial 50 % dilutions of *Flavobacterium pectinovorum* polygalacturonase.

Fig. 4. As Fig. 3. *Erwinia carotovora* polygalacturonase, × ; *Flavobacterium pectinovorum* polygalacturonase, ⊙.

The calcium requirement of the polygalacturonase of *Flavobacterium pectinovorum* was most readily demonstrated viscometrically; increasing calcium concentrations up to 0·01 M resulted in increased enzyme activity. Demonstration of this by the spine-plate technique proved difficult since agar itself is a calcium salt and the addition of yet more calcium only decreased enzyme activity. Silica gel was used instead of agar gel and, provided that the culture fluid itself was rigorously freed from calcium, it could be shown that polygalacturonase activity was negligible in the absence of calcium.

The pH range of the enzyme was studied by the spine-plate and viscometric techniques; the spine-plate test showed a broad optimum between pH 7·4 and 8·2, while the viscometric test revealed an optimum around pH 8·0. At pH 8·5 the control showed a rapid decrease in viscosity which more than offset the slightly increased rate of enzymic action.

The temperature optimum for the enzymic reaction was distinctly higher than that which allowed growth of *Flavobacterium pectinovorum* isolate 81; 37° was optimal but even at 45°, 1/10 of the activity remained. *Erwinia carotovora* 16F was compared with the flavobacterium; for the erwinia the optimum temperature was 40°. Table 3 records these observations.

Table 3. *Temperature relations of the polygalacturonases of* Erwinia carotovora 16F *and* Flavobacterium pectinovorum *isolate* 81

	E. carotovora 16F		*F. pectinovorum* *isolate* 81	
Temp. (°)	Apparent SPU*	% of maximum	Apparent SPU	% of maximum
25	2·7	47	1·3	28
30	3·75	65	2·3	40
37	4·6	79	4·6	100
40	4·8	100	1·8	39
43	1·3	22	0·81	18
45	0·93	16	0·43	9
50	0·16	3	0·13	3

* SPU = spine plate units.

DISCUSSION

The pectic enzymes produced by *Flavobacterium pectinovorum* and *Erwinia carotovora*, and that of *Bacterium aroideae* (Wood, 1955), appear to be of the same general type. A new classification of pectic enzymes has been proposed (Demain & Phaff, 1957). According to this the bacterial polygalacturonases are classified as Endopolymethylgalacturonase II, signifying that they hydrolyse their substrate in a random fashion and attack pectin in preference to pectic acid; the enzyme produced by *Bacterium aroideae* (Wood, 1955) is given as the example of this group. However, all these enzymes readily attack pectic acid, and indeed Wood showed that the *B. aroideae* enzyme hydrolysed pectic acid faster than pectin under his conditions. When a preference for pectic acid is conclusively proved (and providing the culture fluids used do not contain a mixture of polygalacturonases), the enzyme would need to be reclassified as an Endopolygalacturonase II. The very rapid viscosity-decrease of pectin solutions attacked by an enzyme certainly signifies random rather than terminal hydrolysis. The hydrolysis of pectic acid cannot be properly studied in this way since it gels in the presence of the calcium required by the enzyme; however, these gels disperse rapidly (Wood, 1955).

Although the polygalacturonases of *Erwinia carotovora* 16F, and *Flavobacterium* isolate 81 are similar in performance, their mode of production is distinct. It was not found possible to produce the enzyme of isolate 81 in the absence of pectin or pectic acid (i.e. the substrates) or galacturonic acid (the end product of the reaction), but the enzyme of *E. carotovora* 16F was produced constitutively in moderate amounts (up to 20 % of the maximum) in any medium in which it grew actively. Wood (1955) did not grow his organism in a medium containing pectic material, and did not observe whether pectic substances enhanced enzyme production; this may account for his failure to produce pectin methyl esterase. Under optimal conditions *E. carotovora* 16F produced about twice as much enzyme as isolate 81, measured by the spine plate technique.

The production of pectin methyl esterase by *Erwinia carotovora* and the closely related organism, *Bacterium aroideae* (Syn. *Erwinia aroideae*; Rudd Jones, 1950) has been the subject of disagreement. Kraght & Starr (1953) did not prove conclusively that *E. carotovora* produced pectin methyl esterase; the methanol which they found in culture filtrates might have been produced by fermentation of the galacturonic acid. Wood did not find esterase activity in his cultures, but Smith (1958) tested strains of *E. carotovora* and *E. aroideae* and found that they all produced pectin methyl esterase. This has been confirmed in the present work.

The viscometric technique and the hypoiodite method for the determination of reducing groups have been used frequently in polygalacturonase studies (Kertesz, 1951). The spine-plate test is supplementary to these; in effect it measures the amount of sodium pectate which is no longer precipitable by acid after the action of enzyme; its virtue lies in its ease and simplicity, and in the possibility of performing the test on very small amounts of enzyme.

The *Flavobacterium* sp. described in this paper is only one of many types of pectolytic organism present in soil. It is distinguished, however, by its ability to grow on pectic substances as a sole carbon source, and by its inducible polygalacturonase. The absence of a pectin methyl esterase would not prevent the organisms from attacking plant pectins in the soil, since most plant material contains a pectin methyl esterase which de-esterifies the pectin during senescence.

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REFERENCES

DEMAIN, A. L. & PHAFF, H. J. (1957). Recent advances in the enzymatic hydrolysis of pectic substances. *Wallerstein Lab. Commun.* **20**, 119.

DINGLE, J., REID, W. W. & SOLOMONS, G. L. (1953). The enzymatic degradation of pectin and other polysaccharides. II. Application of the 'cup-plate' assay to the estimation of enzymes. *J. Sci. Fd Agric.* **4**, 149.

JANSEN, E. F. & MACDONNELL, L. R. (1945). The methoxyl content of pectins and polygalacturonase. *Arch. Biochem.* **8**, 97.

KERR, A. (1953). A method of isolating soft-rotting bacteria from soils. *Nature, Lond.* **172**, 1155.

KERTESZ, Z. I. (1951). *The Pectic Substances.* New York: Interscience Publishers.

KRAGHT, A. J. & STARR, M. P. (1953). Pectic enzymes of *Erwinia carotovora*. *Arch. Biochem. Biophys.* **42**, 271.

LEES, H. (1949). The soil percolation technique. *Plant & Soil*, **1**, 221.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

PROOM, H., WOIWOD, A. J., BARNES, J. M. & ORBELL, W. G. (1950). A growth-inhibitory effect on *Shigella dysenteriae* which occurs with some batches of nutrient agar and is associated with the production of peroxide. *J. gen. Microbiol.* **4**, 270.

RICHARDS, T. & FOUAD, M. T. A. (1954). The use of glucose-inorganic salts media in the classification of the coli-aerogenes bacteria. II. The indole test and pectate gel liquefaction. *J. appl. Bact.* **17**, Proc. 19.

Rondle, C. J. M. & Carman, B. J. (1956). Agar diffusion plates: the recording of experimental data. *Experientia*, **12**, 443.

Rudd, Jones, D. (1950). On the nomenclature and identity of the coliform soft-rot bacteria. *Trans. Brit. mycol. Soc.* **33**.

Smith, W. K. (1951) The ion-exchange method of preparing silica sols. *Proc. Soc. appl. Bact.* **14**, 139.

Smith, W. K. (1958). A survey of the production of pectic enzymes by plant pathogenic and other bacteria. *J. gen. Microbiol.* **18**, 33.

Weeks, O. B. (1955). *Flavobacterium aquatile* (Frankland and Frankland) Bergey *et al.*, type species of the genus *Flavobacterium*. *J. Bact.* **69**, 649.

Wieringa, K. T. (1949). A method for isolating and counting pectolytic microbes. *Proc. 4th int. Congr. Microbiol., Copenhagen*, p. 482.

Wood, R. K. S. (1955). Studies in the physiology of parasitisms XVIII. Pectic enzymes secreted by *Bacterium aroideae*. *Ann. Bot. Lond.* N.S. **19**, 1.

Zinn, M. K. (1955). *A study on some organic nitrogen compounds of soils.* Ph.D. Thesis, University of Reading.

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Wildy, P. Stoker, M. G. P. & Ross, R. W. (1959). *J. gen. Microbiol.* **20**, 105–112

Release of Herpes Virus from Solitary HeLa Cells

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SUMMARY: A study has been made of the release of herpes virus (strain HFEM) from solitary HeLa cells which had been infected *en masse.* Under the conditions used, small amounts of infective virus were released from a few cells as early as 10 hr. after infection. Evidence is given suggesting that such virus is a remnant of the inoculum. A greater proportion of cells released infective virus (considered to be new virus) between 16 and 34 hr. after infection. The results suggest that: (*a*) the yield of virus is low (average 13 pock-forming units/cell); (*b*) liberation of virus from one cell extends over a period of several hours; (*c*) there is some scatter in the time of release by different cells. The proportion of solitary cells liberating virus 28–35 hr. after infection was lower than expected (19–35 %) considering the virus/cell ratios used, but was not affected by treating the cells with herpes antiserum after they had been exposed to virus. This proportion was compared with the proportion of cells able to initiate pocks on the chorioallantois.

The manner in which animal viruses multiply has become a question of considerable interest, and many studies have been made upon different viruses growing in various host tissues. The use, in such studies, of tissue culture techniques has introduced a fair degree of precision, but because mass cultures of cells have been used in most experiments, little is known about the interactions between virus and individual host cells. There are, however, two reports in which single cells have been studied. In the first (Dulbecco & Vogt, 1954), the growth of western equine encephalomyelitis virus was studied in HeLa cells which had been separated by dilution to a degree which gave a probability of only one cell being present per sample, and in the second (Lwoff, Dulbecco, Vogt & Lwoff, 1955), the growth of this virus was studied in cells which had been singled out under direct vision by the micromanipulation technique of de Fonbrune (1949). As far as we are aware, no other viruses have been studied in this way.

The present paper records a series of experiments dealing with the growth of herpes simplex virus in single HeLa cells, and is similar in approach to that of Lwoff *et al.* (1955). It is one of a series of investigations into the behaviour of this host virus system (Ross & Orlans, 1958; Newton & Stoker, 1958; Stoker & Ross, 1958; Farnham, 1958).

METHODS

The methods of handling HeLa cells and the strain HFEM of herpes simplex virus were given in a previous paper (Stoker & Ross, 1958) where also will be found the composition of media and other solutions used.

Solitary cell cultures. HeLa cells were segregated in small droplets of medium under paraffin oil, a technique which was developed by de Fonbrune (1949) and which we have slightly modified (Wildy & Stoker, 1958). Pyrex glass Petri dishes of 6 cm. diameter were filled with liquid paraffin which had been equilibrated at 37° for at least 24 hr. in an atmosphere of 94 % (v/v) air + 6 % CO_2 and a high relative humidity. Ten small drops of maintenance medium (comprising rabbit serum 5 % (v/v) and lactalbumin hydrolysate 0·5 % (w/v) in Gey's saline) were then disposed upon the bottom of each Petri dish and a pool of about 100 previously infected cells was placed at its centre. The Petri dish was then placed on a microscope stage and all further operations were conducted under direct vision, using a $\frac{2}{3}$ in. objective. One cell at a time was picked from the central pool with a fine Pasteur pipette (about 50 μ diameter) which was connected to the mouth of the operator by means of a flexible tube. The cell was then delivered into a drop of medium. Under these conditions cell division was rarely observed.

Method of titrating infective virus released by solitary cells. At desired intervals after inoculation, the droplets of fluid bathing isolated cells were titrated for infective virus by sucking each up into a pipette and placing it on the chorioallantois of one hen's egg at the 12th day of incubation. The site of the droplet was then washed with a small volume of maintenance medium and this was inoculated on the same egg membrane. Preliminary experiments showed that this procedure recovered at least 90 % of the infective virus which was present in a drop. The chorioallantoic method was used for titration of the HFEM strain because it is more sensitive than plaque counts in HeLa cells (Farnham, 1958).

Stability of viral droplets under paraffin oil. Dr A. E. Farnham (unpublished) has found that suspensions of the HFEM strain of herpes simplex virus in maintenance medium lose about 50 % infectivity in 6 hr. at 37° when kept in glass vessels. It was thought that the infectivity might be affected by the relatively large surface/volume ratio of droplets under oil. This was examined by making a suspension of virus which contained about 500 pock-forming units in 1 ml. maintenance medium. A part of this suspension was incubated at 37° in a test tube and a part as droplets under our experimental conditions.

Infectivity titrations were made at the beginning of the experiment and after incubation for 4 hr. The initial titre of 550 pock units/ml. fell to 280 pock units/ml. when virus was kept in a test tube, and to 230 pock units/ml. when held as droplets under oil. This result is in fair agreement with that of Farnham and, in particular, shows that oil has little, if any, demonstrable effect upon viral infectivity under the conditions of our experiments.

RESULTS

Experiments made in parallel with the mass culture experiments

The experiments were carried out with HeLa cells which had been infected *en masse* during the course of the growth studies of Stoker & Ross (1958), and were intended to furnish supplementary evidence upon the proportion of cells releasing virus. In addition, they have provided some idea of the amounts of new infective virus liberated by solitary cells.

Sheets of HeLa cells, which had been grown in 250 ml. babies' feeding bottles, were washed and exposed to a suspension of virus in maintenance medium at 37° for 1 hr. The ratio number of pock-forming units/number of cells varied from 16 to 79. This is equivalent to 1·2–6·0 HeLa plaque-forming units/cell (cf. Farnham, 1958). The number of plaque-forming units becoming attached to cells was, however, very much less, for under these conditions only about half the virus introduced attaches in 1 hr. (Stoker & Ross, 1958).

The cells were then washed thrice with buffered saline and in four of the seven experiments, medium containing herpes immune serum was left in contact with the cells for 1 hr. After another washing, the serum treatment was repeated and finally, after three further washings, the cells were removed from the glass by a 1/5000 solution of sodium versenate, then suitably diluted with maintenance medium and segregated in droplets as described. In the other three experiments, treatment with immune serum was omitted; instead, the cells were washed nine times with buffered saline.

After incubating the solitary HeLa cells at 37° for periods of 28–35 hr., the droplets were titrated for free infective virus. In parallel, the proportion of pock-forming cells was estimated by the method described previously (cf. Stoker & Ross, 1958). The results may conveniently be described under two subheadings: (*a*) the proportion of cells which liberated virus; (*b*) the amount of virus liberated/cell.

Table 1. *Proportion of HeLa cells which release virus*

The proportion of cells releasing virus was determined by the isolated HeLa cell method and in parallel by inoculating known numbers of HeLa cells on the chorioallantois

				Droplets sampled 28–35 hr. after infection		
Method of removing excess virus	Expt. no.	Virus/cell ratio (pock units/cell)	No. sampled	No. yielding virus	Percentage yielding virus	Pock-forming cells (%)
Washing only	41	22	38	12	32	41
	40	37	69	13	19	90
	33	52	40	14	35	100
			147	39	26·5	
Washing and immune serum	35	16	33	11	30	4
	42	20	72	16	22	<2
	37a	71	19	4	21	11
	34	79	39	10	26	8
			163	41	25	

(*a*) *Proportion of cells which liberated virus.* In Table 1 the proportion of cells which liberated virus into droplets is compared with the proportion of pock-forming cells. It will be seen that the proportion of cells which released virus into droplets varied very little from experiment to experiment (19–35 %), and was related neither to dose of virus/cell (over the small range tested), nor to the use of immune serum. By contrast, treatment with immune serum markedly decreased the proportion of cells which gave rise to pocks (41–100 % with no serum treatment as against <2–11 % after serum treatment). Thus when no immune serum was used, the proportion of cells which formed pocks was always greater than the proportion which released virus into droplets, but after serum treatment the situation was reversed. It is believed that these discrepancies were due principally to the fact that the isolated cell method probably detects infective virus which is released from living cells late during the period of observation, whilst the inoculation of cells on the chorioallantois continuously samples cells and cell debris from the moment that they are placed on the egg membrane. Because of thermal degradation of infective virus, it is unlikely that the former procedure would have detected small amounts of virus which had adhered to, but had not penetrated, cells; the latter method, on the other hand, might be expected to have detected such virus except when it had been neutralized by serum treatment. Supporting this belief, Stoker & Ross (1958) found that about two-fifths of the virus which becomes attached to HeLa cells cannot be washed away, but can be neutralized by antiserum.

These considerations may explain the fallaciously high proportion of pock-forming cells when no antiserum is used, but it is less easy to account for the small number of pock-forming cells after serum treatment. This low value could not well have been caused by residual antibody adsorbed on cells because this would be expected to affect the solitary cells equally. Nor could it have been due to insufficient time having been allowed for a pock to form, because incubation of the eggs for an extra 24 hr. did not increase the number of lesions on the membranes. There remains a further possibility which has not been explored; it is possible that a proportion of HeLa cells which have been inoculated on the chorioallantois perish before they have time to elaborate new virus. If this were so, superficially attached virus on cells which had not been treated with antiserum could still give rise to pocks, but in isolated droplets such virus would decay before the medium was sampled. On the other hand, with serum-treated cells, where little or no superficial virus was present only those cells which released virus would form pocks.

(*b*) *Amounts of virus liberated by individual HeLa cells.* In these seven experiments 80 cells evidently liberated new infective virus. The size of the yield varied widely from cell to cell, as may be seen from Table 2 where the average yield in each experiment and the distribution of individual yields are shown. Taking the overall results, it will be seen that 57 (72 %) of the 80 cells had liberated from 3 to 32 pock units at the time of sampling, and that the average yield/cell was 13 pock units. This value is lower than that found in mass cultures; at the 26th hour Stoker & Ross (1958) found an average yield of 75 pock units/cell; this discrepancy will be discussed later. It will also be seen from Table 2 that the yield of virus was but little affected by serum treatment soon after infection. Although both the average yield and the median yield tended to be higher in the experiments in which no serum was used, the scatter in each experiment was so great that the difference is insignificant. Finally, the results show no relationship between dose of virus and subsequent yield from individual cells, over the range examined.

Table 2. *Numbers of infective virus particles released by individual HeLa cells*

Method of moving excess virus	Expt. no.	Virus/cell ratio (pock units/cell)	Frequency distribution virus yield/cell (pock units)								Total cells yielding virus	Mean yield/cell
			1	2	3–4	5–8	9–16	17–32	33–64	65+		
Washing only	41	22	1	0	1	3	3	4	0	0	12	13
	40	37	3	3	2	2	1	1	1	0	13	9
	33	52	0	0	2	5	3	2	1	1	14	17
		Total	4	3	5	10	7	7	2	1	39	13
Washing and immune serum	35	16	0	2	2	2	1	3	1	0	11	11
	42	20	1	2	3	5	2	1	2	0	16	10
	37 a	71	0	0	1	2	0	0	1	0	4	13
	34	79	3	0	1	1	2	2	0	1	10	17
		Total	4	4	7	10	5	6	4	1	41	13
	Combined totals		8	7	12	20	12	13	6	2	80	13
	%		10	9	15	25	15	16	8	2		

Experiments in which droplets were repeatedly sampled

In six of the experiments described above, droplets were sampled once only at an arbitrary time chosen on the basis of previous experience with mass cultures. Although in each experiment different periods of gestation were allowed before sampling, these varied only over a narrow range. For this reason further experiments were undertaken in which batches of cells were infected and treated with immune serum, as previously described, and the droplets sampled repeatedly. In the first of these (Expt. 41), forty cells were isolated in droplets and were transferred to the hot room at 37°, where all further operations took place. At intervals, the cells were examined and all drops in which cells were still present were tested for infective virus. Virus was also sought in drops where cells had been observed during the previous sampling time; but otherwise where cells had disappeared the droplets were not tested. The results, which are given in Fig. 1, besides providing information upon the time of release of new infective virus, give more evidence on the proportion of cells which liberated virus and the virus yield/cell.

Time of release of new infective virus. It will be seen that in the 40 separate droplets virus was detected in four at the 10th hr., in one at the 13th hr., and in none at the 16th hr. After the 16th hr., the number of droplets containing virus increased. Thus at the 26th hr. four droplets, and at the 33rd 13 droplets,. contained virus. Moreover, it will be noted that at the earlier times the amount of infective virus detected was low (the highest value being two pock units/drop), but later, larger amounts of virus were found (2 to 12 pock units at the 26th hr. and 2 to 17 pock units at the 33rd hr.). These findings strongly suggest that virus detected before the 16th hr. was a part of the inoculum which had become attached to, but had not penetrated, the cells and which had survived washing and serum treatment, whilst that detected later was new infective virus. Hence it appears that new virus was released gradually, beginning in certain cells sometime between 16 and 26 hr., and continuing until the 33rd hr. In a further experiment (Expt. 42) to investigate the possibilities of still later virus release, 6 of 33 cells were found to have liberated virus by the 34th hr. but no virus was detected in the same droplets when these were sampled at the 52nd hr. There was thus no evidence of virus release later than 34 hr. after inoculation.

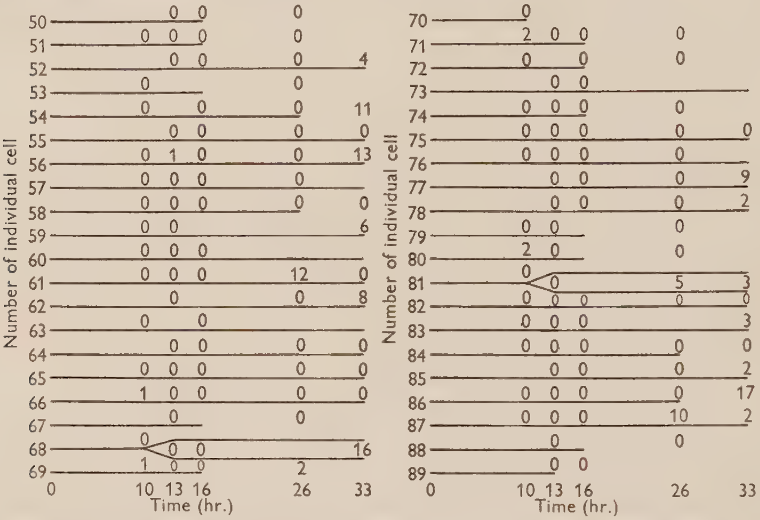

Fig. 1. The history of forty cells which had been exposed to virus, treated with antiserum, washed and finally segregated in droplets. The cells were examined at intervals and the fluid sampled for infective virus. —— = cell still visible, —<, = cell divided. Numbers 0 to 17 = number of pock units of virus detected at any time.

The proportion of cells which released virus. It must be emphasized that the crude values given above may give a poor estimate of the proportion of cells which liberated virus at different times, for two reasons. First, not all cells survived implantation into the drops and, secondly, no allowance has been made for the thermal inactivation of infective virus. Perhaps a better estimate of the proportion of cells which liberated virus at different times may be obtained by neglecting the droplets in which neither cells or virus were found. (Drops containing virus but no cells are included because they imply the earlier presence of a cell.) This procedure gives the following percentages of yielding cells at 10, 13, 16, 26 and 33 hr., respectively: 10, 2·6, 0, 11 and 46 %.

Virus yield/cell. It will be seen in Fig. 1 that 15 cells liberated virus between the 16th and 33rd hours. The values obtained for each cell resemble those found in the earlier experiments (cf. Table 2). They give an average yield of about eight pock units/cell and show considerable scatter from cell to cell. It will be noted that two cells (nos. 81 and 87) which had released virus by the 26th hour gave rise to more virus at the 33rd hr. Though it is possible that the virus detected at the later time was left over from the 26th hr. sampling, this is thought to be unlikely because of the efficiency of washing and thermal inactivation. This, therefore, suggests that individual cells may liberate virus continuously during a period of hours. If this be so, then the sizes of the yields in this experiment may, allowing for thermal inactivation, be at least double the values found.

Other observations. In Fig. 1 it will be noted that two cells (nos. 68 and 81) had divided once by the 13th hr. of the experiment, and subsequently went on to elaborate virus. We have occasionally observed other cells in maintenance medium which divided once and yielded virus, but more recent studies of solitary cells in a growth medium which supports continued cell division, have not shown multiplication of infected cells. It still remains possible that virus may become attached to a cell in the early stages of mitosis, which is then planted as a single cell, and divides before the virus penetrates and begins to grow.

DISCUSSION

When quantitative studies on the growth of virus in mass cultures of cells are attempted, it is necessary to possess a reliable method for determining what proportion of cells become infected. With phage-infected bacteria, the infective centre count fulfils this requirement adequately. The use of the chorioallantois for a similar procedure to determine the proportion of herpes infected HeLa cells is unsatisfactory, because it is impossible to remove by washing all the virus which has become attached to, but not penetrated, the cell. The superficially attached virus will give rise to a pock whether or not the cell is infected. This difficulty may be overcome by treating the cells with antiserum during the latent period, but it is then found that the number of pock-forming cells is less than would be expected, considering the size of original virus inoculum. This may be due to neutralization at the cell surface by residual antibody, or to early disintegration of the HeLa cell on the chorioallantois before virus growth occurs.

The use of isolated cells offers the advantage that residual attached virus is lost by thermal inactivation before the sample is taken for assay, so that antiserum treatment of the cells is not essential. Though fewer cells can be sampled the number is known accurately and the cells may be observed directly. In the series reported about 26 % of cells yielded virus when in solitary confinement. Some cells disappeared without yielding and if these were discounted the proportion releasing virus would be higher, but more recent data suggest that cell disintegration without release may be due to the virus, so these lost cells should probably be included in the total.

The proportion of isolated cells which released virus is higher than that found. by inoculation of antiserum-treated whole cells on the chorioallantois (Table 1). Because antiserum treatment did not affect the proportion of single cells which released virus, it is improbable that residual antibody plays any part in decreasing the number of pocks which result when serum-treated cells are placed on the chorioallantois.

The proportion of isolated cells which release virus is still less than the number which would be expected from the size of the inoculum. In this series of experiments the cells were exposed to between 16 and 79 pock-forming units/cell, which is equivalent to 1·2 to 6·0 HeLa plaque-forming units/cell. Only about half the total plaque-forming units are absorbed in 1 hr. but this should still lead to infection of 45–90 % of the cells. This discrepancy is at present unexplained.

The majority of isolated cells released between 4 and 16 pock units, while Stoker & Ross (1958) estimated that an average of 75 pock units/cell were released by the 26th hr. The disparity may reflect an essential difference in the two methods of sampling liberated virus. Whichever method is used, the values obtained will clearly only approach the truth when cells are sampled at the moment of release or so soon afterwards that thermal inactivation of virus is negligible. When a few cells are each sampled at one instant during the general period of virus release (as with the single cell method), the chances of the sample coinciding with the release period of that cell are remote. On the other hand, when large numbers of cells are used, the system may be regarded as being continuous, and there is a fair probability that a proportion of cells will at any instant of sampling be liberating virus. It seems more probable, however, that the estimate of 75 pock units is too high, because this value was calculated from the total virus detected and the number of cells able to form pocks when placed on the chorioallantois, representing in this case 2·3 % of the cells. In view of the argument in the previous paragraph and of the comparisons shown in Table 1, it seems safe to suggest that this estimate of virus-forming cells is at least ten times too low and, in consequence, the virus yield/cell becomes about 7·5 pock units (or about 0·5 HeLa plaque-forming unit at the 26th hr, which is commensurate with our findings with single cells.

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REFERENCES

Dulbecco, R. & Vogt, M. (1954). One-step growth curve of western equine encephalomyelitis virus on chicken embryo cells grown *in vitro* and analysis of virus yields from single cells. *J. exp. Med.* **99**, 183.

Farnham, A. E. (1958). The formation of microscopic plaques by herpes simplex virus in HeLa cells. *Virology*, **6**, 317.

de Fonbrune, P. (1949). *Technique de micromanipulation.* Paris: Masson & Cie.

Lwoff, A., Dulbecco, R., Vogt, M. & Lwoff, M. (1955). Kinetics of the release of poliomyelitis virus from single cells. *Virology*, **1**, 128.

Newton, A. & Stoker, M. G. P. (1958). Changes in nucleic acid content of HeLa cells infected with herpes virus. *Virology*, **5**, 549.

Ross, R. W. & Orlans, E. (1958). The redistribution of nucleic acid and the appearance of specific antigen in HeLa cells infected with herpes virus. *J. Path. Bact.* **76**, 393.

Stoker, M. G. P. & Ross, R. W. (1958). Quantitative studies on the growth of herpes virus in HeLa cells. *J. gen. Microbiol.* **19**, 250.

Wildy, P. & Stoker, M. (1958). Multiplication of solitary HeLa cells. *Nature, Lond.* **181**, 1407.

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Some Properties of an Inhibitor of Virus Infection from Leaves of Raspberry

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SUMMARY: Raspberry leaves contain a substance which prevents the infection of plants by viruses when it is mixed with the inoculum. It is lost on dialysis, precipitated by nicotine or added protein, unaffected by boiling or freezing, and is probably a phenolic tanning agent. Extracts from peach or apple leaves contain less of such substances than do those from leaves of plum, cherry, raspberry or strawberry. The degree to which virus infection is inhibited by tanning substances from raspberry or other sources depends on the virus and not on the species of test plant. Raspberry tannin and tannic acid combine irreversibly with some viruses but with others the combination is readily reversed by dilution or increase in pH value. With potato ring necrosis, tobacco mosaic and tobacco necrosis viruses, there seems to be a fixed ratio between the amount of virus and the amount of tannin needed to decrease infectivity by a fixed proportion. The infectivity of extracts made from raspberry leaves containing raspberry ring spot or beet ring spot viruses varied with the concentration of tannin present and the pH value. The amount of virus sedimented by centrifuging such extracts at low speed decreased with increase of pH value. Extracts made with 2·5 % nicotine in water were usually more infective than those made with alumina or phosphate buffer (pH 8), and much more infective than those made with water. Both viruses are precipitated by acetone or ammonium sulphate from extracts of infected raspberry leaves made with nicotine or alumina; beet ring spot virus was detected serologically in such preparations from infected raspberry leaves.

Viruses may be difficult or impossible to transmit from plant to plant by mechanical inoculation of sap because of the intrinsic properties of the viruses, because they are present in too small amounts or because extracts from infected leaves contain substances which prevent infection. For example, only a few viruses have been transmitted mechanically from or to rosaceous plants, and it is widely supposed that this is because their leaves contain powerful inhibitors of virus infection and that these are tannins (Bawden, 1956). Bawden & Kleczkowski (1945) showed that leaf extracts made from raspberry and strawberry plants which were infected with aphid-borne viruses contained little or no protein and large amounts of substances, presumed to be tannins, which inhibited infection of plants by viruses and precipitated proteins. However, no attempts seem to have been made to find whether leaf extracts from other kinds of rosaceous plants behave like those from raspberry or strawberry. Much of the evidence for the occurrence of tannins in the saps of rosaceous and other plants quoted in botanical literature is based solely on chemical tests, such as the coloration of metallic salt solutions (e.g. McNair, 1930), which fail to distinguish phenolic compounds that precipitate proteins and possess tanning activity from those that do not. The latter occur in the saps of many plants, for instance tobacco (Reid, 1956), which have no tanning properties and little or no effect on the infectivity of virus preparations.

Tanning agents preserve and stabilize hide, converting it to leather, and it seems generally agreed that they act by linking together different parts of the polypeptide backbone of the same protein fibre and of adjacent protein fibres. Extracts from many kinds of plants possess these properties and also contain phenolic substances, many of which give the diagnostic chemical reactions of 'tannins' used by botanists, although few may actually be tanning agents. White (1957) believed that, of the phenolic substances which occur in plant extracts, only those with molecular weights in the range 500–2000 are tanning agents, and suggested that these limits are set by the need for the molecules to be large enough to have sufficient phenolic groups to cross-link adjacent collagen fibres effectively, yet small enough to diffuse between them. Hide and virus suspensions both contain proteins which react similarly with phenolic substances: the term 'tannin' is therefore used below to denote phenolic substances which possess tanning activity.

Tannins, unlike most substances present in leaf extracts which inhibit virus infection (inhibitors), seem to affect viruses directly and not the host plant (Bawden & Kleczkowski, 1945; Hirth, 1951; Thresh, 1956). As they interfere greatly with the mechanical transmission of viruses from plants that contain them, it is surprising that little is known about their mode of action. My interest in inhibitors of this kind arose from the discovery that raspberry ring spot virus could be transmitted mechanically from raspberry to other hosts when leaves were macerated in nicotine sulphate solutions (Cadman, 1956). Experiments were therefore made to investigate the mode of action of nicotine and to get information on the nature and properties of the inhibitor or inhibitors present in raspberry sap. The results provide new evidence about the reaction between viruses and certain kinds of tannins and suggest various ways of modifying techniques of mechanical inoculation that should be useful in transmitting viruses to and from tannin-containing plants, which include raspberry, strawberry and several kinds of tree fruit plants.

Methods

Leaves from seedlings and plants of commercial varieties of raspberry (*Rubus idaeus* L.) were used in several ways to prepare extracts. Passing leaves through a domestic mincer, squeezing the pulp through muslin and centrifuging the juice for 3 min. at 7000 *g* yielded a non-viscous pale yellow liquid with a highly astringent taste and pH 5·5; this is called raspberry sap. Extracts for chromatography on paper were made either by percolating columns of powdered leaf, dried *in vacuo* over P_2O_5, with 70 % (v/v) acetone in water (Dr A. E. Flood, personal communication); or by extracting minced fresh leaves with 50 % (v/v) acetone in water, straining the pulp through muslin and adding a large volume of ether to the preparation after clarifying it by centrifugation. Chromatograms were run at room temperature in the solvents stated. Phenolic substances were usually detected by developing the papers in the ferric chloride + ferricyanide reagent of White, Kirby & Knowles (1952).

For all tests made with leaves taken from raspberry or strawberry plants which were infected with mechanically-transmissible viruses, and with leaves from other rosaceous plants, weighed amounts of leaf, usually 0·5 g., were macerated in a measured volume of liquid (usually 2·5 ml.) by using a pestle and mortar. When alumina (aluminium oxide, for chromatographic purposes, British Drug Houses, Ltd.) or carborundum were used, weighed amounts were sprinkled over the leaves before they were macerated. The liquids mixed with the leaves were distilled water, buffer solutions or 2·5 % (v/v) commercial nicotine in water (basic alkaloid; 98/99 % distilled nicotine, Murphy Chemical Co.; nicotine sulphate was never used). The preparations made by macerating the leaves were usually further diluted with water before mixing with virus suspensions or inoculating to test plants. For convenience, extracts made with water, alumina or nicotine solution are called water, alumina or nicotine extracts, respectively.

The sources of raspberry ring spot virus (Cadman, 1956) were either fresh sap from systemically infected leaves of *Nicotiana rustica* L., Malling Jewel raspberry or *Fragaria vesca* L. plants; *F. vesca* was infected by grafting with shoots from diseased Malling Promise raspberry (Mr R. M. Lister, private communication). Leaves from naturally infected plants of Malling Seedling V raspberry or frozen sap from infected White Burley tobacco plants, were the sources of the beet ring spot strain of tomato black ring virus (Harrison, 1957, 1959). The Rothamsted culture of tobacco necrosis virus, turnip crinkle virus and potato ring necrosis virus (PRN virus; Cadman, 1958), which is a strain of potato stem-mottle virus, were cultured in French bean, turnip and tobacco, respectively, and the infective saps stored frozen. Most work was done with PRN virus because infection by it was more readily inhibited by tanning agents than was that of the other viruses. Lucerne mosaic virus, and potato viruses *X* and *Y* were used as fresh sap from systemically infected leaves of *Petunia hybrida* Vilm. or *Nicotiana glutinosa* L. Tests with tobacco mosaic virus were made with a preparation from systemically infected White Burley tobacco leaves purified by successive precipitations with ammonium sulphate and at pH 3·2.

The infectivity of different preparations was compared by inoculation to hosts in which countable local lesions were produced. Infectivity tests, using extracts from leaves of raspberry and other plants, tannic acid (British Drug Houses, Ltd.) and other substances, were usually made by mixing preparations with virus suspensions immediately before inoculation. Infectivity of preparations of raspberry ring spot virus, beet ring spot virus, turnip crinkle virus and potato viruses *X* and *Y* was tested in *Chenopodium amaranticolor* Coste & Reyn., that of PRN virus and tobacco necrosis virus in French bean (*Phaseolus vulgaris* L., var. Prince), *Nicotiana glutinosa* and *C. amaranticolor*, and that of lucerne mosaic virus in French bean. The preparations compared were inoculated to equal numbers of whole or half-leaves randomly distributed among the test plants used; at least six whole leaves of *C. amaranticolor*, six half-leaves of *N. glutinosa* and eight half-leaves of French bean were inoculated with each preparation. To increase the numbers of infections, 'Super-floss Celite' (Johns-Manville Ltd.), an abrasive, was usually mixed with the inoculum, which was applied with the forefinger. Leaves were not rinsed after inoculation. The antiserum to beet ring spot virus used in the serological tests was the one described by Harrison (1957).

RESULTS

Properties and nature of the inhibitor from raspberry leaves

Preliminary work showed that sap from roots and leaves of raspberry inhibited infection of French bean by tobacco necrosis virus, that leaf sap was usually more inhibitory than root sap and that its activity varied greatly. Sap from leaves sampled in summer and autumn was more inhibitory than sap from leaves sampled in spring and sap from plants grown under glass in winter contained little or no inhibitor unless the raspberry plants had been exposed for several days to light from mercury vapour lamps.

Tests made with PRN virus in French bean showed that boiling for 10 min. or freezing for 24 hr. had little effect on the inhibitory activity of raspberry sap or leaf extracts (Expt. 1, Table 1). However, leaf extracts made in the presence of nicotine or alumina, and water extracts mixed with an equal volume of healthy tobacco sap or dialysed overnight against tap water had little or no inhibitory activity (Expts. 2 and 4). Similar results were obtained when tobacco mosaic or tobacco necrosis viruses were used instead of PRN virus. Water extracts of raspberry leaves, either freshly prepared or kept frozen or chilled for 48 hr., precipitated the proteins in normal rabbit serum, showing that such preparations cannot be used in serological tests for viruses. However, no precipitates formed when normal serum was mixed at a range of dilutions with freshly prepared extracts made with the aid of alumina or nicotine. The pH value of the extracts was not adjusted before mixing with serum and varied from 5·5 (water extracts) to 7·5 (alumina) and 9 (nicotine). Although the properties of the inhibitor in raspberry leaf extracts suggest that it may be a phenolic substance, they are not peculiar to phenolic substances; stronger evidence is provided by work with acetone extracts of leaves.

Extracts, made by percolating columns of vacuum-dried leaves with 70% (v/v) acetone in water, inhibited infection of French bean by tobacco necrosis virus more powerfully than those made by grinding an equivalent amount of fresh leaf in water. Preliminary work, using paper chromatography and the counter-current distribution technique (Craig, 1944), showed that acetone extracts contained a number of phenolic substances and that inhibitory activity was associated with that part of the extracts which was insoluble in ether. When nine volumes of ether were added to clarified extracts of fresh leaves, made by mixing minced leaves with twice their weight of 50% (v/v) acetone in water, straining the liquid through muslin and centrifuging, material insoluble in ether collected in the aqueous phase of the mixture and could be separated off. The ether and acetone were removed by evaporation under reduced pressure, leaving a clear brown liquid. A cream-coloured sediment, insoluble in water, formed when this liquid was kept for a few hours at room temperature or at 5°. The amount of sediment increased with time and the liquid slowly lost its inhibitory activity. One preparation which, at 1/1000, inhibited PRN virus strongly when first made, had lost 90 % of its inhibitory activity when tested 5 months later, but it still weakly inhibited infection by tobacco mosaic and tobacco necrosis viruses and precipitated normal serum.

Table 1. *Inhibition of potato ring necrosis virus by raspberry sap and leaf extracts*

	Final dilution		Treatment of raspberry sap			
Expt. 1	Virus preparation	Raspberry sap	Fresh	Boiled 10 min.	Aged 24 hr.	Frozen 24 hr.
			Total number of lesions*			
	1/50	1/200	5	36	1	20
	1/50	1/2000	1167	1165	1347	1202
	1/100	0	180		670	
	1/500	0	70		122	
			Treatment of leaf extract			
Expt. 2	Virus preparation	Raspberry leaf extract	Undialysed	Dialysed		
	1/100	1/400	49	1335		
	1/100	1/1600	68	2495		
	1/100	0	2850			
	1/2000	0	64			
			Raspberry leaf extract made with			
Expt. 3	Virus preparation	Raspberry leaf extract	Water	Nicotine†	Alumina†	
	1/50	1/500	0	457	27	
	1/50	1/5000	29	202	234	
	1/100	0	248			
	1/1000	0	21			
			Raspberry sap mixed 1:1 with			
Expt. 4	Virus preparation	Raspberry sap preparation	Water	Tobacco sap		
	1/20	1/200	17	1806		
	1/20	1/2000	1653	—		
	1/100	0	249			
	1/500	0	107			

* Total number of lesions on 8 half-leaves of French bean.

† Preparations adjusted to pH 5·5 before mixing with virus.

Paper chromatography showed that the ether-insoluble fraction contained a number of phenolic substances. The major constituent, however, was one which streaked in N-acetic acid and was immobile in butan-1-ol: acetic acid: water (6:1:2) mixture and in water-saturated butan-2-ol. This substance fluoresced a dull violet in ultraviolet light when the paper was exposed to ammonia vapour, and it coloured ferric salts mauve. This substance is almost certainly a tannin but its identity is unknown. Tests made by Dr D. E. Hathway (personal communication) showed that it did not contain phloroglucinol groups.

When 0·6 ml. samples of the ether-insoluble fraction were applied, as a band of 20 μl. spots to 25 × 30 cm. sheets of Whatman 3MM paper and the sheets run in distilled water, phenolic materials separated into three bands with R_F-values of approximately 0, 0·5–0·6 and 0·7–0·8, respectively. The sheets were subsequently dried, cut transversely into strips 2 cm. wide and these soaked overnight in 3 ml. phosphate buffer (pH 7). The eluates, adjusted to pH 5·5 with 0·1 N-HCl, were mixed with samples of PRN virus preparations already diluted 1/250 with water, and the mixtures inoculated to French bean leaves. In two tests of this kind, the eluate from the strip which included the bulk of the phenolic material with zero R_F value decreased infectivity to 20–25% that of control virus preparations diluted with water and adjusted to pH 5·5, a degree of inhibition comparable to that caused by the original extract diluted 10^{-3} or by a solution containing 0·25 μg. tannic acid/ml. The phenolic material which had an R_F value of zero in organic solvents was probably eluted from the paper in these experiments, but no tests were made to confirm this. Eluates from the band with R_F 0·6–0·7 decreased the infectivity of PRN virus slightly, but those from all other portions of the chromatogram had no such effect.

Although the results do not exclude the existence of other kinds of inhibitors, they suggest that the inhibitory properties of raspberry leaf extracts are associated with one or more phenolic substances. The major phenolic constituent of preparations made from raspberry leaves is probably a tannin which is present in amounts adequate to account for the inhibitory effects observed. The results of comparative tests with tannic acid and raspberry leaf extract suggest that raspberry leaves contain the equivalent of 10–100 mg. tannic acid/g. fresh leaf. However, complex phenolic substances often rapidly undergo chemical changes during isolation from plant tissue, and it is not certain that the inhibitor present in raspberry leaves is identical with the tannin in extracts made from them.

As it is so often stated that the leaves of all rosaceous plants contain tannins, it is interesting to compare the behaviour of raspberry leaf extracts with those from related plants. The results of tests with PRN virus (Table 2) show that peach and apple leaf extracts inhibited infection much less than those from raspberry, strawberry, plum and cherry.

Mode of inhibition of virus infection by tannins

Bawden & Kleczkowski (1945) and Thresh (1956) inferred that raspberry and strawberry leaf extracts inhibited infection of plants by viruses by acting directly on the viruses rather than on the host plants, and they implied that all tannins might behave in this way. Thresh likened the reaction between viruses and tannins to that between hide proteins and tannins, and suggested that viruses form non-infective complexes with tannins, from which infective virus is released when the complexes are dissociated by dilution or increase of pH value. My results support these ideas but they also show the need for caution in making generalizations.

The best evidence that raspberry tannin and tannic acid act directly on

Table 2. *Inhibition of infection of French bean by potato ring necrosis virus by water extracts from leaves of rosaceous plants*

1 vol. of leaf extract was mixed with 1 vol. of virus preparation already diluted 1/50 with water and the dilution factor equivalent to the degree of inhibition observed estimated from dilution curves for PRN virus in water plotted for each experiment.

Source plant	Final dilution of leaf extract	Dilution factor equivalent to degree of inhibition observed
Peach	1/20	1/2·7
Apple	1/20	1/5·6
Raspberry	1/1000	1/3·7
Fragaria vesca	1/100,000	1/2·1
Cherry	> 1/100,000	1/10
Plum	> 1/100,000	1/10

viruses is the fact that the degree of inhibition depends on the virus but not on the host plant (Tables 3 and 4). The figures in column 4, Table 3, were obtained by plotting dilution curves for the different viruses and estimating from these the dilution in water needed to decrease infectivity to the same extent as did the added inhibitor. Infection with PRN virus was strongly inhibited by preparations of inhibitor diluted until they were without effects on infection by the other viruses. Infection of French bean by PRN virus or tobacco necrosis virus was not inhibited by spraying the lower surfaces of leaves with 4 % tannic acid solution at intervals before or after inoculation, by rubbing inoculated surfaces of leaves with tannic acid solution, 50 μg./ml., 30 min. or more after inoculation nor by mixing non-tanning phenolics in the

Table 3. *Inhibition of infection by different viruses caused by raspberry sap or tannic acid*

	Final dilution		Dilution factor equivalent to degree of inhibition observed	
Virus	Virus preparation	Raspberry sap		Test plant
Tobacco necrosis	1/5000	1/5	1/7·4	French bean
Lucerne mosaic	1/50	1/50	1/5·5	
Potato ring necrosis	1/50	1/1000	1/8·1	
Raspberry ring spot	1/25	1/5	1/8·0	*Chenopodium amaranticolor*
Turnip crinkle	1/20	1/5	1/6·3	
	Virus preparation	Tannic acid (μg./ml.)		
Tobacco necrosis	1/30,000	500	1/6·3	French bean
Tobacco necrosis	1/30,000	100	1/2·0	
Potato ring necrosis	1/100	2	1/4·6	
Potato ring necrosis	1/100	0·5	1/1·3	

inoculum. The non-tanning phenolics included quercitrin, D-catechin, chlorogenic, cinnamic, gallic and protocatechuic acids and a leucoanthocyanin from cocoa (kindly supplied by Dr T. Swain) and were used at concentrations of 1 mg./ml.

Table 4. *Inhibition of infection of different hosts by potato ring necrosis virus caused by water extracts of raspberry leaves*

Final dilution		Test plant		
Virus preparation	Raspberry leaf extract	*Nicotiana glutinosa**	*Chenopodium*† *amaranticolor*	French† bean
		Total number of lesions‡		
1/20	1/50	2	5	6
1/20	1/500	386	65	47
1/20	0	759	136	294
1/100	0	305	66	43

* Celite mixed in the inoculum.

† No Celite in the inoculum.

‡ Figures are total lesions in 6 half-leaves of *N. glutinosa*, 6 whole leaves *C. amaranticolor* and 8 half-leaves of French bean.

Thresh (1956) showed that when tannic acid at a concentration sufficient to abolish infectivity was mixed with tobacco mosaic virus, the precipitate produced contained nearly all the virus: infectivity was fully restored by resuspending the precipitate in a volume of water equal to that of the untreated preparations. He obtained similar results when a tobacco necrosis virus from strawberry was mixed with raspberry or strawberry leaf sap, and he showed that the infectivity of non-infective mixtures of viruses and inhibitors was restored when these were diluted with water. My results from similar experiments with potato viruses *X* and *Y*, tobacco mosaic, tobacco necrosis, turnip crinkle and raspberry ring spot viruses confirm and extend his results, except that tobacco necrosis virus was only partially precipitated by concentrations of tannic acid or raspberry leaf extract which completely precipitated the other viruses. Inhibitor was readily detected in the resuspended precipitates from mixtures of tobacco mosaic with tannic acid or raspberry tannin, showing that the non-infective precipitates are mixtures of virus and inhibitor. Precipitates were also produced when raspberry sap or leaf extracts or tannic acid were added in varying amounts to clarified sap from tobacco plants infected with PRN virus, but no virus was detected in the resuspended precipitates; nor was the infectivity of non-infective mixtures of virus and inhibitor restored by dilution. Similar results were obtained when raspberry sap was mixed with sap from *Petunia hybrida* plants infected with lucerne mosaic virus.

The effects of other kinds of inhibitors which precipitate viruses, such as ribonuclease or the glycoprotein from *Phytolacca esculenta*, are also abolished by dilution, but the infectivity of mixtures of virus and inhibitor bears little relation to the proportion of the constituents (Kassanis & Kleczkowski, 1948).

The infectivity of mixtures of PRN virus with raspberry leaf extract, or of tobacco mosaic or tobacco necrosis viruses or PRN virus with tannic acid, however, depends on the ratio of virus to inhibitor. The results in Table 5 show that the concentration of tannic acid needed to decrease the infectivity of preparations of PRN virus by an amount equivalent to a 1/20 dilution with water was proportional to the virus concentration. After centrifugation for 2 min. at 7,000 *g*, the supernatant fluid from mixtures of PRN virus with tannic acid was less infective than comparable uncentrifuged mixtures, but the amount of the decrease seemed independent of the concentrations of the reactants. Similar results were obtained when tobacco mosaic or tobacco necrosis viruses were used instead of PRN virus, but both these viruses are relatively insensitive to tannic acid: at least 500 μg./ml. was needed to halve the infectivity of preparations containing 100 μg./ml. tobacco mosaic virus.

Table 5. *Amount of tannic acid needed to prevent infection of French bean by different amounts of potato ring necrosis virus*

1 vol. of tannic acid solution was mixed with 1 vol. of virus preparation.

Virus content of preparation (arbitrary units)	Concentration of tannic acid needed to decrease infectivity by an amount equivalent to a dilution of 1/20 with water (μg./ml.)	Ratio virus/tannic acid
250	47·2	5·3
50	8·9	5·6
10	1·5	6·7

Tannic acid and raspberry tannin also differ from most other kinds of inhibitors in that their effects increase with increase of time between mixing with viruses and inoculating the mixtures to test plants. In one experiment with PRN virus, virus preparations mixed with 5 μg. tannic acid/ml. 48, 24 and 0 hr. before inoculation to French bean, produced, 35, 92 and 123 lesions, respectively, compared with 909 and 206 produced by untreated virus preparations at the same dilution and at 1/4, of the others, respectively. Thornberry (1935) obtained striking evidence of the delayed reaction of tannic acid with tobacco mosaic virus and showed that the degree of inhibition depends on the pH value; concentrations of tannic acid which abolished or greatly decreased infectivity when mixed with tobacco mosaic virus at pH 4·5 had little or no effect when mixed with virus at pH 7–8. I have noticed that the optical properties of mixtures of tobacco mosaic virus and tannic acid also vary with the pH value of the mixture. Those containing virus and tannin in proportions less than 2:1 showed no anisotropy of flow when viewed in polarized light but this property was regained by mixtures containing 100 μg.virus/ml. or more, when the pH value was raised to 7. Anisotropy of flow was gradually lost when the pH value of mixtures in which the ratio was more than 2:1 was lowered to pH 4·0: those mixtures that contained more than 2 mg. virus/ ml. set spontaneously to birefringent gels. Mixing PRN virus and tannic acid at pH 8·0 decreased but did not abolish the inhibitory effect of the latter.

The results described above strongly suggest that when raspberry sap or tannic acid are used at concentrations which do not injure inoculated leaves, they inhibit infection of plants by viruses through direct action on the viruses.

Factors affecting the mechanical transmission of viruses from raspberry

Plants of Malling Jewel raspberry infected with raspberry ring spot virus and plants of Malling Seedling V infected with beet ring spot virus offered convenient systems for testing the methods for transmitting viruses from tannin-containing plants suggested by the results obtained with *in vitro* mixtures. Maceration of infected leaves of Malling Jewel raspberry in 10 to 20 times their weight of water diluted the tannin sufficiently to give preparations which infected *Chenopodium amaranticolor*, *Petunia hybrida*, tobacco and *Nicotiana rustica*. However, maceration of the leaves in buffer solutions of pH 6–8 gave preparations which were much more infective. The results in Table 6 are typical of many and were obtained by macerating infected raspberry leaves in 0·05 M-phosphate buffer of the specified pH. Raspberry sap is acid, and drops of 0·1 N-NaOH had to be added at intervals to maintain the desired pH value. All the preparations were made up to the same volume with water and then centrifuged at 7000 *g* for 3 min. The precipitates were suspended in volumes of buffer equal to those of the supernatant fluids and the pH values re-adjusted where necessary. Infectivity was much less at pH 5·3 than at 6·9; preparations made at pH 6·9 and 8·0 had similar total infectivities but more of the virus was present in the supernatant fluid at pH 8·0 than at 6·9. Infectivity also increased when the resuspended precipitate and the supernatant fluid at pH 5·3 were adjusted to pH 8·0; indeed the total infectivity then almost reached that of the preparation made at pH 8·0. These effects were independent of the ionic constitution of the buffer solutions and extracts made at pH 8 with dilute NaOH were as infective as those made with buffer. Extracts made with neutral phosphate buffer lost much of their infectivity when dialysed overnight against tap water but little or none when dialysed against neutral buffer; comparable preparations made with water lost more of

Table 6. *The effect of pH on the infectivity of extracts made from leaves of Malling Jewel raspberry infected with raspberry ring spot virus*

The inocula were prepared by macerating 0·5 g. infected leaf tissue in 2·5 ml. 0·05 M phosphate buffer of the specified pH, centrifuging and re-suspending the precipitates in volumes of buffer solution equal to and of the same pH as those of the supernatant fluid.

	pH of extract		
	5·3	6·9	8·0
	Total number of lesions*		
Precipitate	3	1744	871
Supernatant	0	128	1457
Total	3	1872	2328

* Total number of lesions that developed in 6 whole leaves of *Chenopodium amaranticolor* inoculated with the preparations diluted 1/20 and mixed with Celite.

their infectivity than those prepared with buffer when allowed to stand overnight at room temperature and also lost more infectivity when dialysed against water than against buffer.

Extracts made with 2·5 % nicotine solution adjusted to pH 8 with N-HCl were usually more infective than those made with either alumina or phosphate buffer at the same pH value. The ratio of alumina to infected raspberry leaf seemed critical when preparations were made with water but not when made with pH 8 phosphate buffer. Equal weights of alumina and leaf gave more infective preparations and more of the virus was present in the supernatant fluid than when the ratio was 1:5; with increasing amounts of alumina the pH value of preparations made with water increased from 5·3 to 7·5. The abrasive action of alumina seems unimportant, for comparable preparations made with water and equal weights of carborundum or alumina produced 11 and 1735 lesions in six inoculated leaves of *Chenopodium amaranticolor*, respectively. Neither alumina nor nicotine increased the infectivity of extracts from raspberry leaves infected with raspberry ring spot virus made and held at pH 5·3 (Table 7), and at the concentrations tested (1 %) neither cinchonine, which is soluble only in acid solutions, nor colchicine at either pH 5 or 10, increased the infectivity of extracts from infected raspberry leaves. Solutions of nicotine sulphate, which are neutral and decrease in pH value when mixed with acid leaf material, do not behave like nicotine (Mr R. M. Lister, private communication).

Table 7. *The effect of alumina and nicotine at different pH values on the infectivity of extracts made from Malling Jewel raspberry leaves infected with raspberry ring spot virus*

The inocula were prepared by macerating 0·5 g. infected leaf in 2·5 ml. 0·05 M phosphate buffer solution, 2·5 % nicotine or water plus alumina readjusted to the correct pH with HCl or NaOH where necessary. The preparations were centrifuged and the precipitates resuspended in a volume of buffer equal to and of the same pH as the supernatant fluid.

		Expt. 1		Expt. 2	
		Alumina + HCl	Buffer	Nicotine + HCl	Buffer
		Total number of lesions*		Total number of lesions*	
pH 5·3	Precipitate	18	6	54	63
	Supernatant	9	9	0	17
	Total	27	15	54	80
		Expt. 3		Expt. 4	
		Alumina + NaOH	Buffer	Nicotine + HCl	Buffer
pH 8·0	Precipitate	58	194	296	416
	Supernatant	1250	622	2796	934
	Total	1308	816	3092	1350

*** Total number of lesions that developed in 6 whole leaves of *Chenopodium amaranticolor* inoculated with the preparations diluted 1/20 and mixed with Celite.**

The infectivity of extracts made with the aid of alumina or nicotine from raspberry leaves containing raspberry ring spot virus varied little during the growing season, and raspberry yellow dwarf virus (Harrison, 1958) was readily transmitted mechanically from senescent infected leaves of Malling Exploit raspberry collected in October.

Concentrations of nicotine and alumina which increased the infectivity of raspberry sap also increased the infectivity of extracts of *Fragaria vesca* leaves containing raspberry ring spot virus. When 0·5 g. leaf was ground in 5 ml. of water, pH 8 phosphate buffer, 2·5 % nicotine solution or with 0·1 N-NaOH + 0·5 g. alumina, the preparations diluted 1/50 with water and inoculated to six leaves of *C. amaranticolor* produced 14, 361, 1642 and 1410 lesions, respectively.

Harrison (1957) found that beet ring spot virus was precipitated from clarified infective tobacco sap, with little loss of infectivity, by acetone or ammonium sulphate. Both beet ring spot and raspberry ring spot viruses were precipitated when extracts of infected raspberry leaves made at pH 8 with either alumina or nicotine, were mixed with an equal volume of acetone or one half volume of saturated ammonium sulphate solution adjusted to pH 7 with dilute NaOH. Although most of the precipitate did not dissolve in neutral phosphate buffer or water, most of the virus did (Table 8). The most infective preparations were usually made by acetone precipitation from nicotine extracts. That nicotine is more effective than alumina in the extraction procedure is also indicated by the presence of material which inhibited PRN virus in alumina extracts but not in nicotine extracts.

Table 8. *Precipitation of beet ring spot and raspberry ring spot viruses from extracts of infected raspberry leaves*

The inocula were prepared by macerating 1g. infected leaf in 5 ml. 2·5 % nicotine or water made alkaline with dilute NaOH and 1 g. alumina. The ammonium sulphate was adjusted to pH 7 with NaOH and the precipitates produced by this and acetone were resuspended in water. All preparations were tested at equivalent dilutions.

		Raspberry ring spot virus		Beet ring spot virus	
Precipitating agent	Final dilution of sample	Alumina extract	Nicotine extract	Alumina extract	Nicotine extract
		Total number of lesions*			
—	1/20	880	463	1851	3505
—	1/100	167	273	550	469
acetone†	1/20	910	2061	912	2254
ammonium sulphate‡	1/20	894	409	195	1620

* Total number of lesions that developed in 6 leaves of *Chenopodium amaranticolor*.
† 1 vol. acetone added to 1 vol. extract.
‡ 1 vol. saturated ammonium sulphate solution added to 2 vols. extract.

The infectivity of tannin-free preparations from raspberry leaves infected with beet ring spot virus compared favourably with that of sap from infected tobacco plants; attempts were therefore made to detect the virus serologically in the raspberry extracts. A preliminary test showed that the virus could be detected serologically after precipitation with acetone from mixtures of infective tobacco sap and leaf extract from healthy raspberry. No precipitation occurred when the preparation was incubated with normal serum or saline. The results of experiments with infective raspberry leaf extracts were disappointing, however. In one of these, 10 g. leaves, from infected plants of Malling Seedling V were macerated in 25 ml. of 2·5 % nicotine solution, the liquid strained through muslin, clarified by centrifugation and mixed with an equal volume of acetone. The precipitate produced was resuspended in one-fifth of the original volume of water and centrifuged again. This preparation was almost as infective as the nicotine extract and it precipitated specifically with antiserum to beet ring spot virus, although the precipitation end-point with antiserum diluted 1/30 was only 1/1 after 5 hr. incubation at 36°. Infected raspberry leaves may contain less virus than tobacco leaves, or precipitation may be inhibited by other constituents of the preparation from raspberry, which contained little or no detectable tannin but smelt of nicotine. Further work is needed to find whether the technique used can be improved, but the present results show that beet ring spot virus can be detected by serological methods in raspberry leaf extracts.

Both beet ring spot and raspberry ring spot viruses are soil-borne (Harrison, 1956, 1957), have no known aerial vectors and can be transmitted by mechanical inoculation to a great variety of plants other than raspberry. Many attempts were made to transmit aphid-borne raspberry viruses to herbaceous hosts by the techniques described above, but all failed.

DISCUSSION

Many different kinds of substances which inhibit the infection of plants by viruses combine reversibly with and precipitate the viruses, but with most this combination seems irrelevant to their action as inhibitors. As the potency of such inhibitors seems to depend considerably on the identity of the host plant and to be independent of the virus, the ratio of virus to inhibitor in the inoculum and the time of exposure of virus to inhibitor, they are thought to act by altering the susceptibility of the host plant to infection and not by affecting the virus directly. Clearly, neither raspberry tannin nor tannic acid behave in this way. Their effects are independent of the host but depend on the virus; combination between tannin and virus seems always associated with loss of infectivity, and both effects are reversible with some viruses but irreversible with others. There is a fixed ratio between the amount of virus and the amount of tannin required for neutralization of a fixed proportion of infectivity and the degree of inhibition increases with time of exposure of virus to tannin. Both substances therefore seem to inhibit by acting directly on the virus. Further work on the nature of the reaction is needed, but it seems likely that both tannic acid and raspberry tannin act by clumping together the virus particles into complexes which have little or no infectivity. That the infectivity of supernatant fluids from centrifuged mixtures of tannic acid with PRN, tobacco mosaic or tobacco necrosis viruses, was always less than that of comparable uncentrifuged mixtures, suggests that infectious particles may become trapped in the sedimentable virus + tannin aggregates. The failure of some simple phenolic substances to precipitate or to inhibit tobacco necrosis virus suggests that ability to agglutinate virus particles, like ability to stabilize hide, may be a function of the size and molecular structure of the phenolic compounds.

The common usage of the term tannin implies a degree of uniformity in properties and behaviour among tanning agents for which there is little justification and it would be rash to conclude that all tannins react with viruses in the same way as do raspberry tannin and tannic acid.The phenolic tannins which occur in plant extracts fall into two main categories: (i) hydrolysable tannins, such as the gallotannin of tannic acid, which are essentially esters of glucose with gallic and related phenolic acids (White, 1957); (ii) condensed tannins many of which are quinone polymerized phenolics (Hathway, 1958*a*, *b*). It seems agreed that both kinds of tannin form electrovalent linkages with the basic groups and co-ordinate linkages with the peptide groups of hide proteins, and that the relative importance of these varies both with the nature of the tannin and the length of exposure of hide to tannin (Gustavson, 1949; White, 1956). Both kinds of linkage are sensitive to pH but electrovalent links tend to dissociate more readily than co-ordinate ones at pH 7. If the kind of union between viruses and tannins varies to a like degree, then the prospects of preparing infective extracts from tannin-containing plants may depend as much on the nature of the tannin as on the nature, and probably the concentration, of the viruses in such plants. However, many viruses may prove transmissible by mechanical inoculation of sap from tannin-containing plants when leaf extracts which contain relatively little tannin or which are prepared under conditions which prevent the union of tannin with virus are used.

The influence of shade in decreasing the concentration of leucoanthocyanins in leaves has been noticed by Hillis & Swain (1957) and by Holden (1957), and the results of my experiments suggest that the synthesis of tannis in raspberry is also affected by light. If this is a general phenomenon then shading seems worth trying as a means of decreasing the concentration of tannin in plants whose leaves contain large amounts. Since the tannin content of leaves seems to increase during the growing season, young leaves in spring may be the best sources of virus. That leaf extracts from closely related species may differ greatly in tannin content, as for example do plum and peach, suggests that many viruses may prove sap-transmissible after being transferred, by grafting, to related plants whose leaves are relatively tannin-free.

Although alumina removes some and nicotine appears to remove most of the tannin from raspberry leaf extracts, extracts from leaves containing raspberry ring spot or beet ring spot viruses made with buffer solution at pH 8 were as infective as those made with the aid of alumina or nicotine. This is probably because weak solutions of nicotine are strongly alkaline (pH 9–10) and mixing alumina with weakly acid preparations raises their pH value above neutrality. Thus both substances maintain the pH value of leaf extracts to a value where the union of raspberry tannin with these viruses is prevented. Macerating virus-infected leaves in alkaline solutions may therefore be a simple way of increasing the infectivity of extracts from plants containing tannins whose inhibitory action is sensitive to pH values around neutrality.

The results of the serological tests show that extracts made from raspberry leaves with the aid of nicotine are relatively tannin-free and contain protein. Those from leaves infected with beet ring spot virus contained enough virus to be detectable by the precipitation test, suggesting that they may be used successfully for the preparation of antisera to this virus with or without preliminary concentration of the virus by high-speed centrifugation, or precipitation with acetone or ammonium sulphate. These findings probably hold for other sap-transmissible viruses which attain relatively high concentrations in raspberry and perhaps for similar viruses in other tannin-containing plants. Although numerous attempts to transmit aphid-borne viruses mechanically from raspberry to herbaceous test plants all failed, the preparations used may in fact have contained virus which could have been concentrated by high-speed centrifugation and used in attempts to prepare antisera to such viruses.

Little is known of the functions of tannins in plants; the popular belief that they protect plants against invasion by pathogens receives little support from work with plant viruses. It is true that inhibitor is released in detectable amounts when raspberry leaves are rubbed with water and that beet ring spot virus is readily transmissible by mechanical inoculation of infective tobacco sap to peach, a plant which seems free from tannin but not to raspberry, which contains much (Cadman, unpublished). But the presence of tannins in roots and leaves seems to confer no protection on raspberry or strawberry plants against infection by viruses which are transmitted either by insect vectors or through the soil.

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REFERENCES

Bawden, F. C. (1956). Inhibitors and plant viruses. *Advanc. Virus Res.* **2**, 32.

Bawden, F. C. & Kleczkowski, A. (1945). Protein precipitation and virus inactivation by extracts of strawberry plants. *J. Pomol.* **21**, 2.

Cadman, C. H. (1956). Studies on the etiology and mode of spread of Scottish raspberry leaf-curl disease. *J. hort. Sci.* **31**, 111.

Cadman, C. H. (1958). A ring necrosis virus of potato. *Proc. 3rd Conference on potato virus diseases, Wageningen*, p. 168.

Craig, L. C. (1944). Identification of small amounts of organic compounds by distribution studies. II. Separation by counter-current distribution. *J. biol. Chem.* **155**, 519.

Gustavson, K. H. (1949). Some protein-chemical aspects of tanning processes. *Advanc. Prot. Chem.* **5**, 354.

Harrison, B. D. (1956). Soil transmission of Scottish raspberry leaf-curl disease. *Nature, Lond.* **178**, 553.

Harrison, B. D. (1957). Studies on the host range, properties and mode of transmission of beet ring spot virus. *Ann. appl. Biol.* **45**, 462.

Harrison, B. D. (1958). Raspberry yellow dwarf—a solid-born virus. *Ann. appl. Biol.* **46**, 221.

Harrison, B. D. (1959). Further studies on raspberry ring spot and tomato black ring, soil-borne viruses that affect raspberry. *Ann. appl. Biol.* (in the Press).

Hathway, D. E. (1958*a*). An approach to the study of vegetable tannins by the oxidation of plant phenolics. *J. Soc. Leath. Tr. Chem.* **42**, 108.

Hathway, D. E. (1958*b*). Oak-bark tannins. *Biochem. J.* **70**, 34.

Hillis, W. E. & Swain, T. (1957). Influence of illumination on the synthesis of leuco-anthocyanins in leaves. *Nature, Lond.* **179**, 586.

Hirth, L. (1951). Contribution à l'étude de l'inhibition du virus de la mosaïque du tabac par diverses substances tanniques. *Ann. Inst. Pasteur*, **80**, 458.

Holden, M. (1957). An investigation on polyphenolic compounds of the cacao leaf in connexion with a chemical method for detecting virus infection. *J. Sci. Fd Agric.* No. 10, 553.

Kassanis, B. & Kleczkowski, A. (1948). The isolation and some properties of a virus-inhibiting protein from *Phytolacca esculenta*. *J. gen. Microbiol.* **2**, 143.

McNair, J. B. (1930). Gum, tannin and resin in relation to specificity, environment and function. *Amer. J. Bot.* **17**, 187.

Reid, W. W. (1956). The polyphenols and polyphenolase of tobacco, in *Chemistry of Vegetable Tannins. Symp. Soc. Leath. Tr. Chem.* p. 75.

Thornberry, H. H. (1935). Effect of tannic acid on the infectivity of tobacco mosaic virus. *Phytopathology*, **25**, 931.

Thresh, J. M. (1956). Some effects of tannic acid and of leaf extracts which contain tannins on the infectivity of tobacco mosaic and tobacco necrosis viruses. *Ann. appl. Biol.* **44**, 608.

White, T. (1956). The chemical principles of vegetable tannage. *J. Soc. Leath. Tr. Chem.* **40**, 78.

White, T. (1957). Tannins—their occurrence and significance. *J. Sci. Fd Agric.* No. 7, 377.

White, T., Kirby, K. S. & Knowles, E. (1952). Tannins. IV. The complexity of tannin extract composition. *J. Soc. Leath. Tr. Chem.* **36**, 148.

(*Received* 18 *July* 1958)

Gordon, R. E. & Mihm, J. M. (1959). *J. gen. Microbiol.* **20**, 129–135

A Comparison of *Nocardia asteroides* and *Nocardia brasiliensis*

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SUMMARY: A description of *Nocardia asteroides* (Eppinger) Blanchard, based on the examination of 98 strains, is presented and compared with a description of *N. brasiliensis* (Lindenberg) Castellani and Chalmers, based on 50 strains. Characteristics common to freshly isolated strains, variants of the same strain, and old stock strains are used in the delineation of each species.

A taxonomic study of three closely related genera, *Mycobacterium*, *Nocardia* and *Streptomyces*, was begun in 1951 to find a group of stable correlating criteria for the identification of each taxon represented. An attempt was made to define each species in such a way that strains belonging to it could be recognized regardless of their stage of growth or history of cultivation *in vitro*. To effect this, newly isolated strains, variants of the same strain, and strains maintained *in vitro* for 1 to 30 or more years have been examined. Correlating characteristics common to all these strains have been sought for the delineation of the species. This work has been based on a collection now numbering 1254 strains. The collection has not been static, as strains from a variety of sources have been added continually. Newly received strains were examined as soon as possible for observation not only of the reactions of freshly isolated strains, but also of the variations of the older strains due to different conditions of maintenance used by the donors.

Nocardia asteroides (Eppinger) Blanchard, one of the species believed to be represented sufficiently in this collection, was described by Gordon & Mihm (1957). Since the 1957 report, several more strains of this species were found among those obtained from other investigators and are listed here.

Another aggregate of strains, possessing a distinctive group of characteristics, was considered to typify adequately the species *Nocardia brasiliensis* (Lindenberg) Castellani and Chalmers. The strains assigned to this species are described here and compared with those of *N. asteroides*.

METHODS

The observations and tests used in this study were described by Gordon & Smith (1955) and Gordon & Mihm (1957).

Strains used. Nineteen more recently acquired strains of *Nocardia asteroides* are listed in Table 1. At the time of their examination, seven of the 19 were recently isolated strains. These 19 strains bring the total of strains representing the species in this collection to 98. *Nocardia brasiliensis* is represented by the

50 strains listed in Table 3. Ten of these were examined within 6 months of isolation, and a few came from diagnostic laboratories in this country.

Table 1. *Additional strains identified as* Nocardia asteroides (*Eppinger*) *Blanchard*

Laboratory no.	Name when received, source, and strain name or number
747	*Nocardia asteroides* (Eppinger) Blanchard; Shirley McMillen, Hektoen Inst. for Medical Research; A. González Ochoa (652)
785	*N. asteroides*; B. W. Lacey, Westminster School of Medicine (A_{10}, lung)
796, 797	*N. asteroides*; F. C. Kull, Ciba Pharmaceutical Products Inc., Summit, N. J. (282, 341)
842	*N. asteroides*; J. D. Schneidau, Jun., Tulane Univ. (314); ATCC (9970); C. W. Emmons (9956)
850	*N. brasiliensis* (Lindenberg) Castellani and Chalmers; J. D. Schneidau, Jun. (506); C. Lacaz (787)
783	*N. caprae* (Silberschmidt) Waksman and Henrici; B. W. Lacey (A_2); NCTC (659)
722	*Nocardia* sp.; H. A. Waisman, Univ. of Wisconsin (pulmonary nocardiosis)
727, 728, 730	*Nocardia* spp.; L. Ajello, Communicable Disease Center, Chamblee, Ga. (M 170–6, M 171–6, M 173–6); W. M. Bowman
739	*Nocardia* sp.; W. K. Harris, Univ. of Mass. (44, bovine mastitis)
782	*Nocardia* sp.; B. W. Lacey (A_1, ulcerating cervical lymph node)
833, 834	*Nocardia* spp.; Mary P. Lechevalier, Rutgers Univ. (R 17ly, soil; AM 93, soil)
837	*Nocardia* sp.; J. B. Davis, Magnolia Petroleum Co., Dallas, Texas (107–236, utilizes ethane, Davis, Chase & Raymond, 1956)
572	*Proactinomyces asteroides* var. *crateriforme* Baldacci; N. M. McClung, Univ. of Kansas (10); Centraalbur. v. Schimmelcultures
709	*Streptomyces* sp.; S. Froman, Olive View Sanatorium, Calif. (24279, sputum)
830	Unidentified isolation; Charlotte C. Campbell, Walter Reed Army Medical Center (294, drainage from rectal fistula)

RESULTS

Nocardia asteroides (*Eppinger*) *Blanchard*

When received, none of the 19 strains had a specific name not initially borne by one or more of the strains first studied (Gordon & Mihm, 1957, Table 2); a discussion of their nomenclature need not be repeated.

Microscopic appearance. Cultures of 10 of the 19 strains grown on glycerol agar for 5 days at 28° showed branching filaments of varying lengths; seven, a mixture of rods and short or long filaments; one, coccoid forms, rods, and short branching filaments; and one, only thin, curved, lightly staining rods, often containing one or two dark granules. Seven of the 19 strains were not acid-fast. The remaining 12 varied from those which showed an occasional acid-fast rod or filament to those in which 50 % of organisms retained the carbol fuchsin. In some cultures, only portions of the filaments were acid-fast.

Eighteen of the 19 cultures formed loosely filamentous colonies, usually with extensive intertwining substrate (vegetative) hyphae. The colonies of the remaining culture were dense with a halo of short substrate hyphae. Under

Table 2. *Comparison of physiological characteristics*

Property	98 strains of *Nocardia asteroides* (% positive strains)	50 strains of *Nocardia brasiliensis* (% positive strains)
Decomposition of		
Casein	0	98
Gelatin	36	100
Tyrosine	0	100
Xanthine	0	0
Hydrolysis of starch	58	56
Acid from		
Adonitol	0	0
Arabinose	0	0
Erythritol	6	0
Galactose	24	92
Glucose	100	96
Glycerol	98	98
Inositol	2	100
Lactose	0	0
Maltose	6	0
Mannitol	0	94
Mannose	19	68
α-Methyl-D-glucoside	0	0
Raffinose	0	0
Rhamnose	33	0
Sorbitol	0	0
Xylose	0	0
Nitrite from nitrate	85	92
Growth at		
50°	24	0
45°	31	2
40°	90	56
37°	98	100
35°	100	100
28°	100	100
10°	12	30
Utilization of		
Acetate	100	100
Benzoate	0	0
Citrate	33	98
Malate	100	100
Propionate	100	100
Pyruvate	100	100
Succinate	100	98

the conditions of the observation of colonial morphology, the substrate hyphae of only four of the 19 strains fragmented into shorter forms. Aerial hyphae were seen on the colonies of all 19 strains, varying from rudimentary to long and branching. Those of some cultures coalesced, while those of others formed many short side branches, often in clusters. The aerial hyphae of two strains segmented into chains of even bead-like spores (Gordon & Mihm, 1958).

Macroscopic appearance. After 14 days of incubation on yeast glucose agar at 28°, the growth of the 19 cultures varied from thin to abundant, restricted to spreading, flat, finely wrinkled, or warty and heaped-up, beige or peach shading to dark pink. Nine of the cultures were thickly covered with whitish aerial hyphae. The aerial hyphae of six cultures were sparse, while those of the remaining four were invisible to the unaided eye.

Table 3. *Strains identified as* Nocardia brasiliensis (*Lindenberg*) *Castellani and Chalmers*

Laboratory no.	Name when received, source, strain name or number
425, 426	*Actinomyces mexicanus* Boyd and Crutchfield; Isabel Christison, Duke Univ.; N. F. Conant (990, 993); A. González Ochoa (416, 413)
536	*Nocardia brasiliensis* (Lindenberg) Castellani and Chalmers; Isabel Christison; N. F. Conant (2393); M. da Silva (leg lesion)
556	*N. brasiliensis*; E. N. Azarowicz, Univ. of Calif. (104); A. González Ochoa (468)
600–605, 634	*N. brasiliensis*; A. González Ochoa, Inst. de Salubridad y Enfermedades Tropicales, Mexico (439, mycetoma of foot; 448, mycetoma of foot; 463, thoracic mycetoma; 456, mycetoma of knee; 473, thoracic mycetoma; 478, thoracic mycetoma; 414, facial mycetoma)
654–656, 679	*N. brasiliensis*; E. N. Azarowicz (129, 127, 131, 128); Inst. Oswaldo Cruz (2587, 2589, 2588, 2592)
700–702	*N. brasiliensis*; C. W. Emmons, Nat. Inst. of Health, Bethesda, Md. (9960, 9961, 9962); A. González Ochoa (mycetoma)
740	*N. brasiliensis*; J. D. Schneidau, Jun., Tulane Univ. (382); A. González Ochoa
744, 790, 792	*N. brasiliensis*; A. González Ochoa (409, 1002, 1008)
745, 746	*N. brasiliensis*; Shirley McMillen, Hektoen Inst. for Medical Research; A. González Ochoa (410, 416)
748	*N. brasiliensis*; J. E. Mackinnon, Inst. de Higiene, Montevideo, Uruguay (IHM 824); C. Lacaz
749, 750	*N. brasiliensis*; J. E. Mackinnon (IHM 984, IHM, 1045); A. González Ochoa (thoracic mycetoma, madura foot)
798–800	*N. brasiliensis*; A. González Ochoa (4014, thoracic mycetoma; 4015, mycetoma of foot; 4016 ankle lesion)
844	*N. brasiliensis*; J. D. Schneidau, Jun. (327); A. Batista (586)
845	*N. brasiliensis*; J. D. Schneidau, Jun. (381); A. Batista (631); Inst. Pasteur, Paris (337)
846–849	*N. brasiliensis*; J. D. Schneidau, Jun. (385, 386, 386B, 505); C. Lacaz (95, 85, 85, 253)
851	*N. brasiliensis*; J. D. Schneidau, Jun. (507, cutaneous lesion)
W 3485–W 3488	*N. mexicana*; S. A. Waksman, Rutgers Univ.; A. González Ochoa (409, 413, 414, 415)
405	*N. mexicana*; Julia M. Coffey, N.Y. State Dept. of Health (47296); J. B. Fischer; N. F. Conant
432	*N. mexicana*; Isabel Christison; N. F. Conant (2178); A. González Ochoa
555	*N. mexicana*; E. N. Azarowicz (73); Lucille K. Georg (149)
688	*N. mexicana*; L. Ajello, Communicable Disease Center, Chamblee, Ga. (149); N. F. Conant (992)
N 2391	*N. pretoriana* Pijper and Pullinger; NCTC (2391)
731	*Nocardia* sp.; L. Ajello (M 174–6); W. M. Bowman
733	*Unidentified isolation*; Charlotte C. Campbell, Walter Reed Army Medical Center (650)
774 A, 774 B	Unidentified isolations; Charlotte C. Campbell (variants of 8016, submandibular abscess)

Physiological characteristics. The physiological properties of the 19 more recently acquired strains were similar to those of the 79 previously studied strains of *Nocardia asteroides*. Characteristics of all the 98 strains are presented in Table 2. One of the two strains that did not grow at 37° was isolated from soil; the source of the second strain is unknown. Two of the nine strains that grew at 37° but not at 40° were obtained from human infections; five, from soil; and two were received without history of isolation. The following physiological criteria were considered the most important for the separation of strains of *N. asteroides* from those of other species of *Nocardia* and those received as streptomycetes: failure to attack casein, tyrosine, or xanthine; inability to form acid from adonitol, arabinose, erythritol, inositol, lactose, maltose, mannitol, α-methyl-D-glucoside, raffinose, sorbitol, or xylose; and non-utilization of benzoate. For the separation of strains of *N. asteroides* from senescent strains of all species of aerobic actinomycetes, which rarely give positive results to physiological tests, the following characteristics were used: acid production from glycerol and glucose, and the use of acetate, malate, propionate, pyruvate, and succinate as carbon sources. Variation by a strain from the pattern of these more dependable reactions in only one respect did not hinder its identification as *N. asteroides*.

Nocardia brasiliensis (*Lindenberg*) *Castellani and Chalmers*

The strains received as *Actinomyces mexicanus* Boyd & Crutchfield (1921), *Nocardia brasiliensis* (Lindenberg, 1909) Castellani & Chalmers (1913), and *N. pretoriana* Pijper & Pullinger (1927) were believed to agree with their original specific descriptions. The transfer of *A. mexicanus* and *N. pretoriana* to the synonymy of *N. brasiliensis* by González Ochoa (1945) and González Ochoa & Sandoval (1956) was confirmed. We were not able to find authority for the assignment of the species *mexicanus* of Boyd & Crutchfield (1921) to the genus *Nocardia*. Ota (1928), the authority usually cited, does not mention the species or the report of Boyd & Crutchfield. A search for effective publication of *N. mexicana*, therefore continues.

Microscopic appearance. After 5 days of growth at 28° on glycerol agar, 37 of the 50 cultures showed long branching filaments. The remaining 13 cultures consisted of coccobacilli, short to long rods, and short branching filaments. Nine of the cultures were not acid-fast, and the rest ranged from a few to 100 % acid-fast forms. Only portions of the filaments of approximately one-third of the cultures retained the carbol fuchsin.

All but two of the strains assigned to this species formed loosely or densely filamentous colonies or both. The substrate (vegetative) hyphae were usually long, rhizoid, intertwining, and were not observed to fragment. Two of the strains presented dense colonies with compact margins and densely filamentous colonies. All strains produced aerial hyphae that varied from rudimentary to short, straight or gnarled, or long and branching. The aerial hyphae of nearly half of the strains tended to coalesce. Round or irregular masses were scattered throughout the aerial hyphae of nine strains. Sporulation was not observed under the conditions of the examination. It is conceded, however, that failure to produce spores might have been due to the use of media unsuitable for sporulation by these strains.

Macroscopic appearance. As with *Nocardia asteroides*, the appearance of the cultures on yeast glucose agar after 2 weeks of incubation at 28°, was one of the most mutable characteristics of the strains. The growth of some of the cultures was light yellow to yellowish orange, dry, spreading, finely wrinkled, and without aerial hyphae visible to the unaided eye. The growth of these strains might easily be mistaken for that of some mycobacteria. The growth of other cultures was beige to yellowish orange, spreading, thickly coated with whitish aerial hyphae, wrinkled, cracked, and identical in appearance with cultures received as *Streptomyces rimosus*. The remaining cultures ranged between the two extremes with beige, yellow, or yellowish orange, wrinkled, spreading growth. Some were sparsely covered with aerial hyphae, and a few formed amber to brown soluble pigment.

Physiological characteristics. The reactions of the 50 strains representing *Nocardia brasiliensis* are listed in Table 2 in comparison with those of *N. asteroides*. The following properties were deemed the most useful in the separation of strains of *N. brasiliensis* from those of *N. asteroides*: decomposition of casein and tyrosine and acid formation from inositol and mannitol. For the division of strains of *N. brasiliensis* from the strains of the remaining species of *Nocardia* and from strains received as streptomyces, the following criteria were the most important: failure to decompose xanthine; inability to produce acid from adonitol, arabinose, erythritol, lactose, maltose, α-methyl-D-glucoside, raffinose, rhamnose, sorbitol, or xylose; and non-utilization of benzoate. Acid formation from glucose and glycerol and the use of acetate, citrate, malate, propionate, pyruvate, and succinate as carbon sources provided positive characteristics in the pattern of reactions of this species. Deviation in one respect from this series of reactions was permitted in the identification of strains as *N. brasiliensis*.

DISCUSSION

The purpose of this study in seeking to identify old stock strains and variants as well as freshly isolated strains of each species is admittedly different from that of the diagnostic laboratory. Certain criteria which are reliable in the diagnostic laboratory, such as acid-fastness of freshly isolated strains of *Nocardia asteroides* and *N. brasiliensis*, must be accepted as variable when older strains are considered. Many of the tests and observations used here, on the other hand, require more time than is available to the diagnostician. In spite of the diversity in viewpoint and methods, the conclusions of this study confirm those of Almeida & Lacaz (1941), González Ochoa (1945), Moore, Lane & Gaul (1954), and others, in the recognition of *N. asteroides* and *N. brasiliensis* as two distinct species.

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REFERENCES

ALMEIDA, F. DE & LACAZ, C. S. (1941). Estudo sobre o *Actinomyces brasiliensis* (Lindenberg, 1909). *Ann. Fac. Med. S. Paulo*, **17**, 577.

BOYD, M. F. & CRUTCHFIELD, E. D. (1921). A contribution to the study of mycetoma in North America. *Amer. J. trop. Med.* **1**, 215.

CASTELLANI, A. & CHALMERS, A. J. (1913). *Manual of Tropical Medicine*, 2nd ed. p. 816. New York: William Wood and Company.

DAVIS, J. B., CHASE, H. H. & RAYMOND, R. L. (1956). *Mycobacterium paraffinicum* n.sp., a bacterium isolated from soil. *Appl. Microbiol.* **4**, 310.

GONZÁLEZ OCHOA, A. (1945). Estudio comparativo entre *Actinomyces mexicanus*, *A. brasiliensis* y *A. asteroides*. *Rev. Inst. Salubr. Enferm. trop., Méx.* **6**, 155.

GONZÁLEZ OCHOA, A. & SANDOVAL, M. A. (1956). Revision determinativa de algunas especies de actinomicetes patogenos descritas como diferentes. *Rev. Inst. Salubr. Enferm. trop., Méx.* **16**, 17.

GORDON, R. E. & MIHM, J. M. (1957). A comparative study of some strains received as nocardiae. *J. Bact.* **73**, 15.

GORDON, R. E. & MIHM, J. M. (1958). Sporulation by two strains of *Nocardia asteroides*. *J. Bact.* **75**, 239.

GORDON, R. E. & SMITH, M. M. (1955). Proposed group of characters for the separation of *Streptomyces* and *Nocardia*. *J. Bact.* **69**, 147.

LINDENBERG, A. (1909). Un nouveau mycétome. *Arch. Parasit., Paris*, **13**, 265.

MOORE, M., LANE, C. W. & GAUL, L. E. (1954). Nocardiosis of the knee caused by *Nocardia brasiliensis*. *Arch. Derm. Syph., N.Y.* **70**, 302.

OTA, M. (1938). Champignons parasites de l'homme. (Études morphologiques et systematiques). *Jap. J. Derm. Urol.* **28**, 16, 381.

PIJPER, A. & PULLINGER, B. D. (1927). South African nocardiases. *J. trop. Med. (Hyg.)*, **30**, 153.

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SPICER, C. C. & DATTA, N. (1959). *J. gen. Microbiol.* **20**, 136–143

Reversion of Transduced Antigenic Characters in *Salmonella typhimurium*

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SUMMARY: A strain of *Salmonella typhimurium*, in which the H antigens had been altered by transduction with a bacteriophage lysate of *S. abony* to the form *b* ↔ *enx* has been found to revert in the first or second phases giving rise to organisms with the structure *i* ↔ *enx* and *b* ↔ 1.2. No reversions in both phases have so far been detected. This phenomenon has been observed only in non-lysogenic strains; lysogenic varieties have remained stable.

Transduction by bacteriophage of antigenic characters in the salmonella group was first reported by Zinder & Lederberg (1952); a more detailed study of the subject was published by Lederberg & Edwards (1953). It was shown that flagella antigens could be freely transferred between members of groups A, B, and D of the Kauffmann-White scheme, Phase I antigens of the donor being transduced to the Phase I locus of the recipient, and Phase II antigens, independently, to the Phase II locus. Many salmonella types were 'synthesized' by transduction, including several not yet observed in nature. The synthetic types were carefully examined for any sign of the continued presence of the recipient's original H antigen, and none was found. The new types appeared as stable as naturally occurring salmonellae, and no reversions to the parent type were discovered.

Later Edwards, Davis & Cherry (1955) were able to extend these studies to other groups of the Kauffmann–White scheme; they likewise did not report any reversions in their transformed cultures. It has therefore been assumed that the genetic factor controlling the H antigen has been completely replaced by the transduced gene, and that the original fragment has been eliminated. It is the purpose of this note to give an account of observations on some salmonella H antigens which have been changed by transduction, and subsequently reverted to the parent form.

METHODS

The main salmonella strains used are listed in Table 1. All were obtained from Dr B. A. D. Stocker and Dr C. Quadling of the Lister Institute, London, S.W. 1, and those whose reference initials are SL had been prepared by them.

The phage used throughout for transduction was P 22, as described by Zinder & Lederberg (1952). None of the salmonella strains in Table 1 carried P 22, the SL series having been specially prepared to act as donors in experiments on transduction.

The rabbit sera used for identifying antigens were those prepared for general use by the Standards Laboratory, Colindale. In serum selection experiments

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using semi-solid agar (Edwards & Bruner 1942) the sera were used at a concentration about 200 times their agglutinating titre. Antigenic variants were isolated by inoculating a thick suspension of the culture on semi-solid agar containing antiserum to the H antigens other than those sought. When verifying the phases of an organism, serum selection was done in Craigie tubes (Mackie & McCartney, 1953). For transduction, 1 ml. of a thick suspension (*c.* 1×10^{10} organisms) of an overnight culture was mixed with 1 ml. of a high titre phage preparation, incubated at 37° for ½ hr., centrifuged, and the deposit plated on semi-solid agar containing the appropriate serum.

Table 1. *Origin and antigenic type of salmonella strains used*

	H antigens		O antigens (antigen I omitted)	
Ref. No.	Ph. I	Ph. II		Origin and remarks
LT2	*i*	1.2	4.5.12	A wild strain of *Salmonella typhimurium* (Zinder & Lederberg, 1952)
SL270	*i*	1.2	4.5.12	A double mutant of LT2, requiring methionine and tryptophan
SW803	*b*	*enx*	4.5.12	A strain of *S. abony* catalogued as no. 103 by Edwards & Bruner (1942)
SL113	*b*	1.2	4.5.12	LT2 altered by transduction with a P22 lysate of SW803. Prepared Nov. 1953
SL141	*b*	*enx*	4.5.12	SL113 altered by transduction by the same lysate of SW803. Prepared Jan. 1954
SL147	*b*	*enx*	4.5.12	A streptomycin resistant mutant of SL141, isolated by selection in Jan. 1954
SL150	*b*	*enx*	4.5.12	An azide resistant mutant of SL147, isolated by selection in Feb. 1954
SW715	*r*	1.2	4.5.12	A strain of *S. heidelberg* catalogued as No. 16 by Edwards & Bruner (1942)
SW541	(*i*)	(1.2)	4.12	A non-motile strain of *S. typhimurium.* (Stocker, Zinder & Lederberg 1953)
SW543	(*b*)	(–)	4.5.12	A non-motile monophasic strain of *S. paratyphi* B. (Stocker, *et al.* 1953)

Standard techniques as given by Adams (1950) were used in all work involving bacteriophage.

RESULTS

The main experimental findings are summarized in Table 3 which gives details of the most important strains tested for reversion of transduced antigenic characters to the parent type. The first observations were made in October 1956, when strain SL150 (see Table 1) was plated on nutrient agar and the resulting overnight growth was, as a control in another experiment, inoculated heavily on to a semi-solid agar containing antiserum to factors *b* and *enx*. Strain SL150 was a derivative of *Salmonella typhimurium* LT2, whose H antigens had been changed by two successive transductions with a P22 lysate of *S. abony* to the form *b* ↔ *enx* and which was streptomycin- and azide-resistant. It had been prepared 3 years earlier and the subculture now used had been stored on Dorset egg for one year at room temperature in the dark. A number of swarms developed from it, which were at first taken to be either contaminants or mutants having modified *b* or *enx* antigens. However, further investigations showed them to have the antigenic formula: H; *i* ↔ *enx*, 0; 4, 5, 12, and to be streptomycin- and azide-resistant. No such organism had ever been known in the laboratory and as the possibility of contamination was ruled out, they were taken to be reversions to the original 1st phase H antigen. At that time the *i* variants were in a very small minority and could only be detected by serum selection from a mass culture from the Dorset egg slope. All single colonies tested had the expected H antigens *b* ↔ *enx* and did not give rise to *i* forms on serum selection. Cultures of SL113, SL141 and SL147, from which SL150 had been derived, were examined in the same way. A small proportion of *i* ↔ *enx* forms was detected in SL141 and SL147, those from the former being resistant to neither streptomycin nor azide, and those from the latter being resistant to streptomycin, but not azide, as expected; SL113 remained entirely *b* ↔ 1.2. Single colonies from SL141, 147 and 150, all with H antigens *b* ↔ *enx*, were stored on Dorset egg slopes at room temperature and at 4°, to see whether they would subsequently develop *i* forms.

In November 1956 a similar series of transductions and selections was made starting from *Salmonella typhimurium* LT2, and were labelled SC113, SC141, SC147 and SC150. These cultures differed from the SL series in being lysogenic for phage P22. No *i* forms could be demonstrated in any of these, and they too were stored on Dorset egg slopes at room temperature and at 4°. The SL and SC series of cultures were examined at intervals for 6 months and no changes were seen.

In June 1957 a detailed study of all available cultures of SL113, SL141, SL150 was begun. These consisted of: (*a*) the Dorset egg slopes in which the *i* variant had first been found; (*b*) a duplicate set of Dorset egg slopes kept by Dr Stocker at the Lister Institute; (*c*) a set of subcultures held by Dr E. S. Anderson of the Central Enteric Reference Laboratory since 1956 when he had phage-typed them; (*d*) the single colony subcultures kept at room temperature and at 4° since the previous year; (*e*) a clone from a single cell of strain SL150, isolated by micromanipulation by Dr Quadling at the Lister Institute, in January 1955, and kept on Dorset egg at 4° ever since. In none of these cultures were any organisms found carrying the *b* antigen. Platings direct from the slopes were invariably in the 2nd phase. The 1st phase was isolated by serum selection in Craigie tubes but, as a rule, only after more than 24 hr. incubation; it was in all cases found to be *i*. The second phase was unchanged, as were the O antigens, phage type and streptomycin and azide resistance.

In addition, seven strains of SL150 made colicinogenic by Dr Hamon in November 1956 at the Lister Institute were examined. Three of these had reverted *in toto* in the second phase and four in the first, but none had reverted in both. All were streptomycin-resistant but in some the azide resistance had declined.

At the same time the SC series of cultures was examined. No change in antigenic or other characters was found.

Lysates of members of the SL series prepared at various dates by Dr Stocker and by ourselves were tested for their ability to transduce H antigens and motility to other salmonellas of the same O group. It was found that all the lysates (see Table 2) could transduce motility to SW541, a non-motile strain of *Salmonella typhimurium*, and to SW543, a non-motile variant of *S. paratyphi B* in which the gene determining absence of flagella is closely linked to that controlling the phase I antigen (Stocker, Zinder & Lederberg 1953). Lysates of the unreverted strains failed to transduce the Phase I antigen to *S. typhimurium* LT 2 or to *S. heidelberg* but lysates made after reversion to antigen *i* would transduce that antigen both to *S. heidelberg* and to strain SW543. As it was known that the recipient strains, *S. typhimurium* LT2 and *S. heidelberg*, were in a state in which transductions of antigens in either phase would be manifest (Lederberg & Iino 1956) it can be concluded that the lysates of unreverted strains could not transduce the first phase antigen. However, as it was not known which phase the unreverted strains were in at the time of lysis, no conclusions can be drawn as to the transducibility of the second phase antigen. Lysates of strains which have reverted in the second phase have not yet been tested.

Table 2. *Phage 22 lysates tested for ability to transduce 1st phase antigens to* Salmonella heidelberg *or* S. typhimurium LT2. *All these lysates transduced motility to SW541 and SW543*

Strain lysed	Ref. no.	Date prepared	Titre in *pfu**/ml.	Transduction of Phase I H antigen
SL113	G27	30 Nov. 53	3×10^9	Nil
SL141	G68	21 Jan. 54	5×10^9	Nil
SL147	G73	2 Feb. 54	5×10^9	Nil
SL150	G86	2 Mar. 54	2×10^8	Nil
SL150	G87	2 Mar. 54	1×10^8	Nil
SL150	G137	9 Dec. 54	2×10^9	Nil
SL150	C1	Nov. 55	5×10^9	Nil
SL150†	C2	July 57	5×10^9	+
SD7	C3	May 58	2×10^9	+
SD13	C4	May 58	1×10^{10}	+
SD14	C5	May 58	1×10^{10}	+

* *pfu*, plaque forming units.
† Made after reversion of 1st phase antigen.

In August 1957 a third series of cultures was prepared from strain LT2 by transduction with a new P22 lysate of strain SW803. Again no attempt was made to pick non-lysogenic organisms and the first of the series, $b \leftrightarrow 1, 2$, named SD113, carried phage P22. When SD113 was treated with the same lysate, swarms developed whose centres were clear and which were found to have the H antigens $b \leftrightarrow enx$ and to be non-lysogenic. One swarm was subcultured and labelled SD141. Strain SD147 was a streptomycin-resistant variant of SD141. The antigens of this series were not re-examined until October 1957 when it was found that the lysogenic SD113 remained $b \leftrightarrow 1.2$ but both SD141 and 147, which were non-lysogenic, had become entirely $i \leftrightarrow enx$, their O antigens and streptomycin resistance being unaltered. The reverted form of SD147 was altered by transduction to the form $b \leftrightarrow enx$ using the original lysate of *Salmonella abony* (SW803). This culture is lysogenic and has remained stable.

As all non-lysogenic strains of these three series had reverted to antigen *i* and none of the lysogenic strains had done so, it seemed that these two factors were associated and an attempt was made to build up another non-lysogenic series to study the process in more detail.

Strain SL270, a double mutant of LT2 requiring tryptophan and methionine, was used as a starting-point to provide extra markers for the recognition of contaminants. Some difficulty was encountered in making non-lysogenic stocks but two sets of lysogenic analogues of SL113 and SL141 were prepared between December 1957 and February 1958. These were designated SD 3, 4, 5 and 6, as set out in Table 3.

In March–May 1958 non-lysogenic analogues of SL113 and 141 were prepared, designated SD 7, 13 and 14, the two latter corresponding to SL141, and the former to SL113. No reversions have so far been observed in either the lysogenic or non-lysogenic stocks stored on Dorset egg slopes for 3 months.

P22 lysates of SD7, 13 and 14 differ from lysates of the original reverting SL series in being able to transduce both *b* and *enx* antigens to LT2.

We have tested 22 other non-lysogenic strains of Salmonella (obtained from Dr Stocker and from Dr J. Lederberg of Wisconsin) whose H antigens had been altered by transduction and which had been stored on Dorset egg at room temperature, and in none have we found any sign of antigenic instability.

A non-lysogenic strain of *Salmonella heidelberg* (SW715) prepared in November 1957, using the original lysate of *S. abony* to alter the H antigens to the form $b \leftrightarrow 1.2$, has so far shown no reversions.

In case there should be a natural tendency for a few individual organisms with H antigen *i* to appear among stocks of Salmonella with H antigen *b*, an investigation was made of old stock cultures of *Salmonella paratyphi B* which had been kept on Dorset egg at room temperature for various lengths of time up to 14 years without subculture, and no *i* forms could be demonstrated in any of them.

DISCUSSION

The interpretation of the findings reported above is still obscure. Further work is in progress, but so far no method of controlling the occurrence of reversion has been found, and the necessary experiments are consequently slow. It is not known whether all members of a clone eventually revert or

Table 3. *Summary of information on the most important strains tested for reversion of transduced antigenic characters*

Strain	Date prepared	Parent		Donor		Original H antigens		Reversions			Lysogenicity for P22
						Ph. I	Ph. II	Date	Ph. I	Ph. II	
SL113	Nov. 53	LT2	*i*–1.2	SW803	*b*–*enx*	*b*	1.2	Aug. 57	*i*	.	–
SL141	Jan. 54	SL113	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	Aug. 56	*i*	.	–
SL147	Jan. 54	SL141	*b*–*enx*	.	.	*b*	*enx*	Aug. 56	*i*	.	–
SL150	Feb. 54	SL147	*b*–*enx*	.	.	*b*	*enx*	Aug. 56	*i*	.	–
SC113	Oct. 56	LT2	*i*–1.2	SW803	*b*–*enx*	*b*	1.2	.	.	.	+
SC141	Oct. 56	SC113	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	.	.	.	+
SC147	Oct. 56	SC141	*b*–*enx*	.	.	*b*	*enx*	.	.	.	+
SC150	Nov. 56	SC147	*b*–*enx*	.	.	*b*	*enx*	.	.	.	+
SD113	Aug. 57	LT2	*i*–1.2	SW803	*b*–*enx*	*b*	1.2	.	.	.	+
SD141	Aug. 57	SD113	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	Oct. 57	*i*	.	–
SD147	Sept. 57	SD141	*b*–*enx*	.	.	*b*	*enx*	Oct. 57	*i*	.	–
SL341	Nov. 56	SL150	*b*–*enx*	Colicinogenic from	*S. sonei* P9	*b*	*enx*	Before Aug. 57	.	1.2	–
SL342	Nov. 56	SL150	*b*–*enx*		*S. sonei* P9	*b*	*enx*		.	1.2	–
SL343	Nov. 56	SL150	*b*–*enx*		*E. coli* 3609	*b*	*enx*		.	1.2	–
SL344	Nov. 56	SL150	*b*–*enx*		*E. coli* 235	*b*	*enx*		*i*	.	–
SL345	Nov. 56	SL150	*b*–*enx*		*E. coli* 235	*b*	*enx*		*i*	.	–
SL346	Nov. 56	SL150	*b*–*enx*		*E. coli* 235	*b*	*enx*		*i*	.	–
SL347	Nov. 56	SL150	*b*–*enx*		*E. coli* 235	*b*	*enx*		*i*	.	–
SD1	Nov. 57	SW715	*r*–1.2	SW803	*b*–*enx*	*b*	1.2	.	.	.	–
SD2	Nov. 57	SD147	*i*–*enx*	SW803	*b*–*enx*	*b*	*enx*	.	.	.	+
SD3	Nov. 56	SL270	*i*–1.2	SW803	*b*–*enx*	*b*	1.2	.	.	.	+
SD4	Dec. 57	SD3	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	.	.	.	+
SD5	Feb. 58	SL270	*i*–1.2	SW803	*b*–*enx*	*b*	1.2	.	.	.	+
SD6	Feb. 58	SD5	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	.	.	.	+
SD7	Mar. 58	SL270	*i*–1.2	SW803	*b*–*enx*	*b*	1.2	.	.	.	–
SD13	May 58	SD7	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	.	.	.	–
SD14	May 58	SD7	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	.	.	.	–
SD15	May 58	SD7	*b*–1.2	SW803	*b*–*enx*	*b*	*enx*	.	.	.	+

whether only a fraction do so which subsequently outgrow the rest. However, the reported instability is not due to an error in technique or to contamination, and merits some discussion since the main problem of the genetics of transduction is the mechanism by which the transduced fragment is incorporated into the permanent genetic make-up of the recipient.

So far no examples have been published in which transduced characters in Salmonella have reverted to the original form and the only analogous case that has been worked out in any detail is that of a series of genes influencing galactose fermentation in *Escherichia coli* K12 (Morse, Lederberg & Lederberg, 1956). It was found in *E. coli* K12 that the transduced Gal^+ genes can persist and be replicated in the host cell along with the original Gal^- allele of the recipient without being incorporated in the genetic structure of the host. During growth of bacteria there may be crossing over between the transduced fragment ('exogenote') and the main chromosome. Organisms which are partial diploids in this sense are unstable and frequently revert to the haploid state, but it appears that the supernumerary transduced fragment can be reproduced through a long series of cell divisions. Such a situation could account for the present findings if it be postulated that the transduced gene is in some way dominant to the original one which is still present.

The Gal system provides an analogy in which the original and transduced characters are reproduced together over many cell divisions. However, the transduced Gal character appears to be closely associated with the presence of prophage, while in the present case there is no necessary association of this kind, since the organisms which reverted are non-lysogenic for the transducing phage and fully sensitive to it.

Another important difference between the Gal system and the present one is that the lysates of strains carrying transduced Gal genes will themselves transduce it, whereas lysates of antigenically unstable strains before reversion do not transduce the Phase I antigenic characters, though they can transduce others. This suggests that there is some chromosomal aberration present. Lysates made after reversion has occurred, transduce the Phase I antigen characters with normal efficiency.

An alternative explanation of reversion to the original antigenic structure is mutation. It might be argued that the original *i* character was part of an essentially stable genotype and that mutative reversions to it would be favoured by natural selection. Some such process may well be responsible for the observed stability of the naturally occurring Salmonella types. The main objections to this hypothesis are, first, that no such mutation has been observed in any of the other transduced H antigen characters examined; second, it offers no explanation of the fact that transduced characters in lysogenic strains appear to be stable; and third, that there is no close analogy with mutation from presence of a character to its absence; in the present case the mutation is from synthesis of one protein antigen to another one, antigenically quite different. In addition the independent reversions from *b* to *i* in many separate cultures over a period of eight months indicates a reversion rate higher than would be expected for a mutation.

Neither explanation gives an adequate reason why reversion has so far only been observed at one locus in any one sub-line of SL 141, and not at both, and neither will account for the total disappearance of the non-reverted type.

We are greatly indebted to Dr B. A. D. Stocker, not only for his generous gifts of cultures and bacteriophage, but also for much advice and encouragement during this investigation. We should also like to thank Professor J. Lederberg for supplying some cultures. Phage typing of some strains of *Salmonella typhimurium* was kindly carried out for us by Dr E. S. Anderson of the Enteric Reference Laboratory, Colindale.

REFERENCES

Adams, M. H. (1950). Methods of study of bacterial viruses. *Meth. Med. Res.* **2**, 1.

Edwards, P. R. & Bruner, D. W. (1942). Serological identification of Salmonella cultures. *Circ. Ky Agric. Exp. Stn*, no. 54.

Edwards, P. R., Davis, B. R. & Cherry, W. B. (1955). Transfer of antigens by phage lysates with particular reference to the *l*, *w* antigens of Salmonella. *J. Bact.* **70**, 279.

Lederberg, J. & Edwards, P. R. (1953). Serotypic recombination in Salmonella. *J. Immunol.* **71**, 232.

Lederberg, J. & Iino, T. (1956). Phase variation in Salmonella. *Genetics.* **41**, 743.

Mackie, T. J. & McCartney, J. E. (1953). *Handbook of Practical Bacteriology*, 9th ed. London: E. and S. Livingston Ltd.

Morse, M. L., Lederberg, E. M. & Lederberg, J. (1956). Transduction in *Escherichia coli* K 12. *Genetics*, **41**, 142.

Stocker, B. A. D., Zinder, N. D. & Lederberg, J. (1953). Transduction of flagellar characters in Salmonella. *J. gen. Microbiol.* **9**, 410.

Zinder, N. D. & Lederberg, J. (1952). Genetic exchange in Salmonella. *J. Bact.* **64**, 679.

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Evidence for the Occurrence of Permeases for Tricarboxylic Acid Cycle Intermediates in *Pseudomonas aeruginosa*

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SUMMARY: Washed suspensions and cell-free extracts of *Pseudomonas aeruginosa* grown on Lemco agar, to which intermediates of the tricarboxylic acid cycle had been added, were tested for their ability to oxidize succinate, fumarate, malate, pyruvate, acetate, α-oxoglutarate and citrate. Whole organisms had lag periods of 2–3 hr. before citrate was oxidized rapidly, except when citrate or acetate had been added to the growth medium. There were lag periods of about 10 min. before rapid and linear oxidation of succinate by organisms grown on acetate; of fumarate by organisms grown on acetate or pyruvate; and of acetate by organisms grown on malate, pyruvate, α-oxoglutarate, fumarate or succinate. There were no lags for malate, pyruvate or α-oxoglutarate by organisms grown on any of the substrates tested. Organisms grown on malonate or Lemco agar to which no additions had been made had lags for all the substrates. Glucose-grown organisms had lags for all the intermediates except succinate. Only malonate-grown organisms oxidized malonate rapidly and linearly, and organisms grown without malonate had lag periods of 2–3 hr. before oxidizing this substrate.

Cell-free extracts from organisms with lag periods before the oxidation of citrate, acetate, fumarate, malate, succinate and α-oxoglutarate were shown to oxidize these substrates without a lag period. Pyruvate and malonate were not oxidized by any of the extracts including those from organisms grown on these substrates. Chloramphenicol inhibited the adaptation to substrates by whole organisms but was without effect on the oxidation by cell-free extracts. The significance of these findings in relation to the hypothesis of specific permeases for the transport of organic molecules into the cell is discussed.

Studies on the kinetics of induction of *Escherichia coli* β-galactosidase (Rickenberg, Cohen, Buttin & Monod, 1956) led to the hypothesis that the presence of an inducer, besides producing the specific enzyme β-galactosidase, also resulted in the production of a specific protein (β-galactoside-permease) which enabled the organisms to accumulate β-galactosides. In a review Cohen & Monod (1957) suggested that most, if not all, organic molecules may enter the cell by the action of similar highly stereospecific permeases. We were thus led to consider that the observed oxidation of some of the intermediates of the tricarboxylic acid cycle by extracts, but not by whole bacteria, might be due to the absence of the necessary permeases.

Kogut & Podoski (1953) and Barrett & Kallio (1953) showed that cell-free extracts of *Pseudomonas* spp. oxidized certain intermediates of the tricarboxylic acid cycle rapidly and linearly, whereas with whole organisms there was a lag period before a linear oxidation rate was reached. Both groups of workers suggested that the lag period for whole organisms might involve making the substrate accessible to the enzyme, either across the cell membrane or to some special site within the cell. We thought that the lag periods might be due to the synthesis of the specific permeases required to transport the substrates into the cell. The experiments with *Pseudomonas aeruginosa* described in this paper were designed to test this hypothesis by comparing the oxidative capacities of cell-free extracts and whole organisms grown with different substrates, and the effect of possible inhibitors of the adaptive process. Some of these results were presented in a preliminary report (Meadow & Clarke, 1958).

METHODS

Cultivation of bacteria. Pseudomonas aeruginosa was obtained from the National Collection of Type Cultures as NCTC 8203. Stock cultures were maintained on slopes of Lemco agar (10 g. peptone; 10 g. Lab. Lemco; 5 g. NaCl; 10 g. agar; water to 1 l.; pH 7·4). The inoculum was grown for 6 hr. at 37° with shaking, in tubes of Lemco broth (composition as above without agar). For experiments in the Warburg apparatus, organisms were grown in Roux bottles for 18 hr. at 37° on the surface of 100 ml. Lemco agar to which various intermediates of the tricarboxylic acid cycle or glucose or malonate, had been added to give a final concentration of 0·4 % (w/v). One tube of a 6 hr. subculture in Lemco broth was used to inoculate each Roux bottle. After growth the organisms were washed off the agar with distilled water, filtered through gauze to remove particles of agar, harvested by centrifugation and washed with 50 ml. distilled water. The organisms from two Roux bottles were then resuspended in 20 ml. of 0·05 M-phosphate buffer (pH 7·4) and diluted approximately tenfold for the manometric experiments. In different experiments the amount of organisms used varied from equiv. 5 to 20 mg. dry wt./ml.

Preparation of cell-free extracts. These were prepared by forcing the undiluted suspensions (equiv. *c.* 200 mg. dry wt./ml.) through a small aperture at 10,000 lb./sq.in. in a bacterial press modified from that described by Milner, Lawrence & French (1950). Cellular debris was removed by centrifugation at 20,000 ***g*** for 5 min. and the supernatant fluid used for the Warburg estimations.

Manometric methods. Oxygen uptake was measured by the conventional Warburg technique at 37° with air as the gas phase and 0·2 ml. 40 % (w/v) potassium hydroxide in the centre cup. The main compartment of the Warburg vessel contained 1 ml. cell-free extract or diluted suspension of organisms, 0·2 ml. of inhibitor when used and enough 0·05 M-phosphate buffer to give a total volume of 3·0 ml.; the substrate (0·4 ml. of a 4 %, w/v, solution) was added from the side arm after equilibration. For experiments on malonate decarboxylase, the potassium hydroxide was omitted from the centre well and the experiments were carried out in 0·05 M-phosphate buffer (pH 6·1) in an atmosphere of nitrogen.

Reagents. Chemicals were all of 'Analar' grade when available. The buffer used throughout was Sørenson's phosphate buffer (0·05-M, pH 7·4 and 6·1).

The tricarboxylic acid cycle intermediates, glucose and malonate were obtained from British Drug Houses Ltd., the 8-azaguanine and nitroarginine from L. Light and Co., penicillin (Na salt) from Glaxo Ltd. and chloramphenicol from Parke Davis Ltd.

RESULTS

Comparison of whole organisms and cell-free extracts

The organisms were grown on Lemco agar to which various intermediates had been added at 0·4 % (w/v). The rates of oxidation of these compounds by washed suspensions, and the occurrence of lag periods before the maximum rate of oxidation was reached, depended on which compound had been added to the medium. Kogut & Podoski (1953), using a fluorescent pseudomonad isolated from soil, Barrett & Kallio (1953), using *Pseudomonas fluorescens*, and Stone & Wilson (1952), using *Azotobacter vinelandii*, had shown that although whole organisms had lag periods before oxidizing some of the tricarboxylic acid cycle intermediates there was no lag period with cell-free extracts. With *Pseudomonas aeruginosa* we found similar though not identical results.

A typical series of results, in this case with malate-grown organisms, is shown in Fig. 1 (*a*–*d*). The washed suspensions oxidized malate, succinate, pyruvate and α-oxoglutarate at a linear rate from the time of adding the substrate (Fig. 1*a*). Acetate was oxidized linearly only after a lag period of about 10 min. (Fig. 1*b*) and citrate was only oxidized rapidly after a lag period of about 3 hr. (Fig. 1*c*). Cell-free extracts, prepared with the bacterial press, oxidized citrate, succinate, fumarate, malate, acetate and α-oxoglutarate rapidly with no initial lag period (Fig. 1*d*). The rate of citrate oxidation of these extracts was greater even than the rate of oxidation of malate, the substrate on which the organisms had been grown. These results are in agreement with the view that the lag period represents an adaptation which is concerned with the permeability of the organism to the substrate rather than the adaptive production of the oxidative enzymes.

The effect of chloramphenicol

The lag period was difficult to detect when it was of very short duration and the initial rate of oxidation in the first 5 min. was above the endogenous respiration. Kogut & Podoski (1953) showed that the adaptation to maximum rate of oxidation could be prevented by inhibiting protein synthesis by ultraviolet irradiation. Barrett & Kallio (1953) were able to inhibit this adaptation with amino acid analogues. It was thought that chloramphenicol should also prevent the adaptation, and should make it easier to detect lag periods of 5–15 min. Accordingly, the rates of oxidation of the substrates were measured in the presence and absence of chloramphenicol. When there was a lag before a linear rate of oxidation was established, a clear difference was seen between the rates of oxidation with and without chloramphenicol (60 μg./ml.). When no lag was detected for a particular substrate then its rate of oxidation

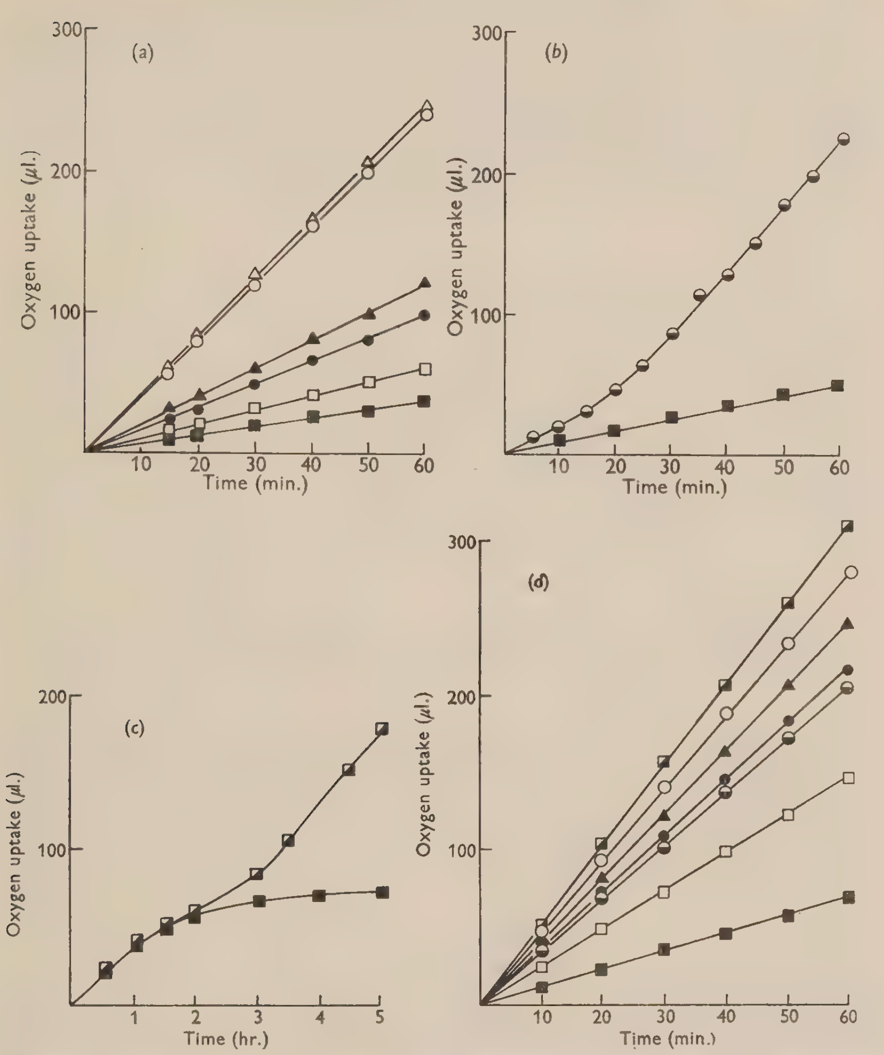

Fig. 1*a–d*. Oxidative activity of malate-grown *Pseudomonas aeruginosa*. (*a*) Whole organisms, △—△, pyruvate; ○—○, succinate; ▲—▲, malate; ●—●, fumarate; □—□, α-oxoglutarate; ■—■, endogenous. (*b*) Whole organisms, ◒—◒, acetate; ■—■, citrate and endogenous. (*c*) Whole organisms, ◪—◪, citrate; ■—■, endogenous. (*d*) Cell-free extracts, ◪—◪, citrate; ○—○, succinate; ▲—▲, malate; ●—●, fumarate; ◒—◒, acetate; □—□, α-oxoglutarate; ■—■, endogenous. (50 μmole substrates.)

was unchanged by the addition of chloramphenicol. Figures 2*a*, *b* show typical results with organisms grown on pyruvate. Pyruvate, succinate, malate and α-oxoglutarate were oxidized linearly from the moment of tipping and the rate was not altered by the addition of chloramphenicol (Fig. 2*a*). Fumarate and acetate gave short lag periods of about 10 min. and there was a marked difference in the rates of oxidation of these substrates in the presence and absence of chloramphenicol (Fig. 2*b*).

Batches of organisms were grown with each of the substrates in turn, and tested in this way for lag periods and for differences in the rate of oxygen uptake with and without chloramphenicol. Whenever a lag period (however short) was observed, there was a clear-cut difference to be seen by adding chloramphenicol. The rate of oxidation by cell-free extracts was unchanged by the addition of the same concentration of chloramphenicol.

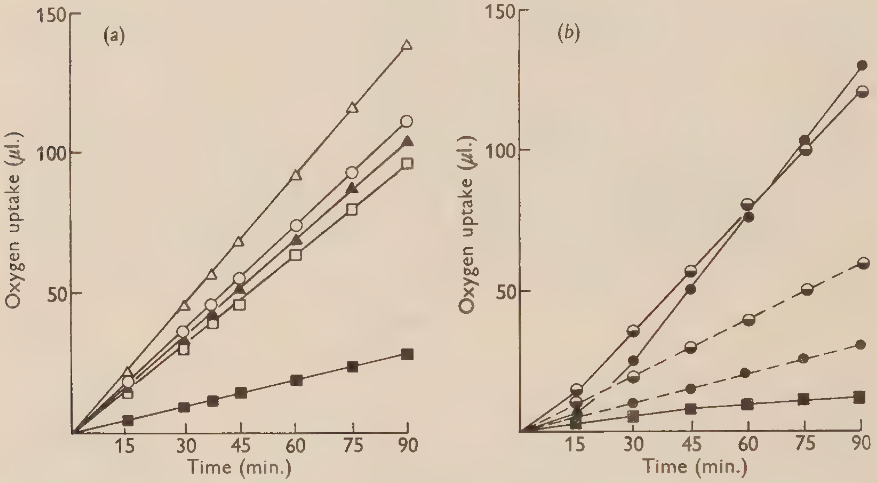

Fig. 2*a*, *b*. Effect of chloramphenicol on the oxidative activity of pyruvate-grown *Pseudomonas aeruginosa*. (*a*) Substrates with rates unchanged in the presence of chloramphenicol, △—△, pyruvate; ○—○, succinate; ▲—▲, malate; □—□, α-oxoglutarate; ■—■, endogenous. The lines represent rates in the presence and absence of 60 μg. chloramphenicol/ml. (*b*) Substrates with adaptation inhibited by chloramphenicol, ———, no chloramphenicol; - - - -, with 60 μg. chloramphenicol/ml.; ●—●, fumarate; ◒—◒, acetate; ■—■, endogenous. (50 μmole substrates.)

Oxidation of citrate

The oxidation of citrate represented a special case. Unless citrate or acetate had been added to the growth medium, there was a period of 2–3 hr. before citrate was oxidized at an appreciable rate. None of the other substrates had such long lag periods; but Stone & Wilson (1952) observed lag periods of about an hour in the oxidation of malate and succinate by *Azotobacter vinelandii* grown on sucrose. It was thought that the short periods of adaptation occurred when the appropriate permease is deficient but not absent. Substrate will then enter the cell fast enough for a full complement of permease to be established quickly. In the case of citrate, the lag period was prolonged to 2–3 hr. and the oxidation rate was hardly above the endogenous during this time. This suggests that the organism was almost completely devoid of citrate-permease; citrate then entered only very slowly and it took a long time for the necessary energy to be made available for protein synthesis. We thought that if this were so, it should be possible to decrease the period of adaptation before the oxidation of citrate by providing at the same time a small amount of a substrate which was known to enter the cell and to be oxidized rapidly. Figure 3 shows the effect of the addition of 1 μmole fumarate together with 50 μmole citrate on the citrate lag period. It can be seen that the lag period was shorter when 1 μmole fumarate was present, and that rapid linear oxidation occurred after 90 min. instead of 180 min. Chloramphenicol inhibited the adaptation to citrate both in the presence and absence of fumarate. Fumarate therefore can be regarded as providing energy for permease synthesis, rather than acting as a 'sparker' of the tricarboxylic acid cycle.

Growth medium and oxidation rate

Table 1 summarizes the results obtained for the oxidation of various substrates by organisms which had been grown on media to which one of these substrates had been added. The washed suspensions were all tested with and without chloramphenicol to confirm short lag periods. The Q_{O_2} values are given for a single batch of organisms on each substrate, but at least two batches of organisms were grown on each substrate, and although the batches varied in activity, the relative rates of oxidation of the various substrates by organisms grown on the same medium appeared to be the same and the same lag periods were found.

Table 1. *The oxidation of tricarboxylic acid cycle intermediates by washed suspensions of* Pseudomonas aeruginosa *grown with these substrates in the medium*

Substrate in growth medium (0·4 %, w/v)	Q_{O_2}							
	Citrate	Acetate	Malate	Pyruvate	α-oxo-glutarate	Fumarate	Succinate	No sub.
Citrate	27	24	35	32	7	32	29	3·3
Acetate	14	20	32	29	10	[45]	[45]	4
Malate	(14)	[23]	17	22	10	16	22	4
Pyruvate	(24)	{29}	28	38	16	[62]	36	9
α-oxoglutarate	(21)	[53]	42	29	43	56	57	6·8
Fumarate	(24)	[20]	21	64	31	44	28	17
Succinate	(19)	[21]	42	40	12	51	43	5·6

Brackets indicate lag periods before this rate was reached. ( ), 2–3 hr. lag; [ ], 10 min. lag; { }, 5 min. lag.

The cell-free extracts prepared with the bacterial press were much more active than those prepared by shaking with glass beads in the Mickle apparatus. There was always a loss of activity as compared with whole organisms and, as might be expected, a variation in the oxidative activity of different extracts. Particular attention was paid to the activity of extracts from organisms which had shown lags towards certain substrates, and all those tested oxidized the substrate linearly irrespective of any lags found with whole organisms. None of the extracts prepared oxidized pyruvate, not even those from organisms grown on pyruvate; occasional preparations had lost the ability to oxidize acetate; and, as will be discussed later, none of the extracts oxidized malonate. No attempt was made to stimulate the oxidation of these substrates by the addition of supplementary co-factors.

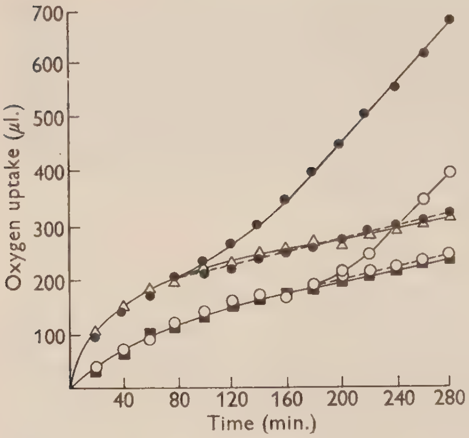

Fig. 3

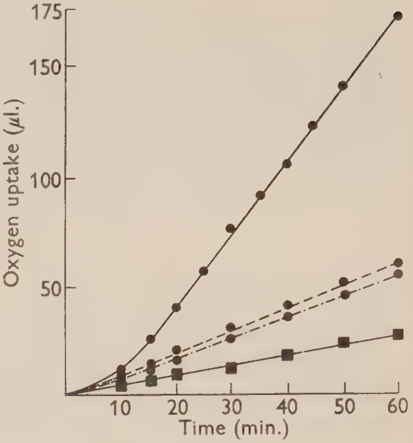

Fig. 4

Fig. 3. The effect of fumarate on the adaptation of fumarate-grown organisms to citrate oxidation. ●—●, 50 µmole citrate + 1 µmole fumarate; ●- - -●, 50 µmole citrate + 1 µmole fumarate + 60 µg. chloramphenicol/ml.; ○—○, 50 µmole citrate; ○- - -○, 50 µmole citrate + 60 µg. chloramphenicol/ml.; △—△, 1 µmole fumarate; ■—■, endogenous.

Fig. 4. The effect of 8-azaguanine on the adaptation of pyruvate-grown organisms to fumarate oxidation. ●—●, fumarate; ●- - -●, fumarate + 60 µg. chloramphenicol/ml.; ●-·-·-●, fumarate + 200 µg. 8-azaguanine/ml.; ■—■, endogenous. (50 µmole fumarate used.)

Effect of growth on other media

Organisms were also grown on Lemco agar with no additions, and on Lemco agar to which 0·4 % (w/v) glucose or sodium malonate had been added. The results are given in Table 2. It can be seen that organisms grown on Lemco agar had lags for all the substrates tested. Extracts from these organisms oxidized all the substrates tested except malonate and acetate. Organisms grown on glucose had lags for all the substrates except glucose and succinate, while organisms grown on malonate had lags for all substrates except malonate. None of the extracts tested, including those from malonate-grown organisms,

Table 2. *Adaptation to the oxidation of tricarboxylic acid cycle intermediates by washed suspensions of* Pseudomonas aeruginosa *grown on glucose and malonate*

Cells grown on Lemco agar with 0·4 % (w/v) substrate	Oxidation of substrates							
	Citrate	Acetate	Malate	α-oxoglutarate	Fumarate	Succinate	Glucose	Malonate
0	L*	L°	L*	L*	L*	L*	NT	L°
Glucose	L*	L	L	L	L	+	+*	L°
Malonate	L	L	L	L	L	L	NT	+°

+ = no lag = permease present; L = lag; NT = not tested; * = extracts tested and active; ° = extracts tested and inactive.

oxidized malonate. Gray (1952) found that washed suspensions of *Pseudomonas aeruginosa* could only oxidize malonate without a lag when they had been grown on malonate. He also showed that malonate-grown organisms could decarboxylate malonate, and that organisms and cell-free extracts, which had lost their ability to oxidize malonate, still had an active malonate decarboxylase. Dried organisms and cell-free extracts prepared from control organisms, grown without malonate, had no malonate decarboxylase activity. Gray (1952) and Wolfe, Ivler & Rittenberg (1955), working with *Pseudomonas fluorescens*, suggested that the oxidation of malonate was preceded by decarboxylation. Hayaishi (1954) and Wolfe, Ivler & Rittenberg (1954, 1955) showed that coenzyme A was involved and proposed that acetyl coenzyme A was formed.

In an experiment carried out in a nitrogen gas phase so that there could be no oxidation of the substrate, we found that whole organisms and cell-free extracts of *Pseudomonas aeruginosa* grown on malonate were able to decarboxylate malonate. The decarboxylase activity was very low and the CO_2 output was less than a twentieth of the oxygen uptake of the same batch of organisms with malonate in air. Fumarate-grown organisms and cell-free extracts prepared from them did not decarboxylate malonate.

Effect of other inhibitors

Penicillin at 600 units/ml. had no effect on the initial rate of oxidation of acetate by acetate-grown organisms and did not inhibit the short adaptation (10 min.) to fumarate or the long adaptation (2–3 hr.) to citrate. At this high concentration the rate of acetate and fumarate oxidation decreased after 60 min., but this was thought to be a non-specific effect.

Nitroarginine was added to the organisms in a few experiments, but had no effect either on the oxidation rate or the adaptation. 8-Azaguanine inhibits the formation of some adaptive enzymes (Creaser, 1956). When it was added to pyruvate-grown organisms at 300 μg./ml. it had the same effect as chloramphenicol in inhibiting the adaptation to fumarate and citrate (Fig. 4). It was thought that this was probably due to inhibition of protein synthesis by interference with nucleic acid synthesis. However, when 8-azaguanine was tested with other substrates, the results were more complex. The oxidation of glucose by glucose-grown organisms was initially inhibited by the addition of 8-azaguanine; the oxidation of succinate by glucose-grown organisms, which oxidized succinate linearly, was not affected for the first 30–40 min., but after that there was a marked increase in the rate of oxidation above that for succinate alone. This increase in the rate of oxygen uptake with succinate and 8-azaguanine only occurred when excess (50 μmole) succinate was present. At low succinate concentrations (1–3 μmole) the rate fell to that of the endogenous after all the succinate had been oxidized. Organisms tested with 8-azaguanine alone had no oxygen uptake over the endogenous even after 3 hr. Oxidation of guanine by glucose-grown organisms occurred after an initial lag period. The fate of 8-azaguanine in these systems is being investigated further.

DISCUSSION

The experiments gave results described in agreement with the earlier findings of Kogut & Podoski (1953) and Barrett & Kallio (1953) with other pseudomonads, that the adaptive phase observed in the oxidation of some of the intermediates of the tricarboxylic acid cycle is due to factors concerned with cellular permeability. We agree with Cohen & Monod (1957) in considering that this adaptive phase is due to the production of specific permeases, the function of which is to enable these compounds to be transported into the cell. We interpret our results as follows. If the washed suspensions can oxidize a compound at a linear rate from zero time, then the cell already possesses an adequate amount of the specific permease for that compound; if there is a lag period of 5–15 min. before the linear rate of oxidation starts, then the cell already contains a little permease, but the amount is so low as to make the rate of entry of substrate into the cell the rate-limiting step in its oxidation; if the initial rate of oxidation remains little more than the endogenous although the extracts contain the relevant oxidative enzyme, then the necessary permease is considered to be absent. We are therefore interpreting the lag periods in these experiments as the period during which the necessary permeases are synthesized. We found that permease synthesis is inhibited by chloramphenicol and probably also by 8-azaguanine, although, as we have shown, care must be taken in the interpretation of the 8-azaguanine experiments since it has more than one effect in the systems we have described. Penicillin, on the other hand, appears to have no effect on permease synthesis in these systems. The evidence therefore depends on the presence or absence of lag periods and demonstration that protein synthesis is involved. The effect of fumarate in shortening the lag periods with citrate provides reasonable evidence that energy is required for permease synthesis. More rigorous proof for the existence of these permeases would be provided if it could be shown that the compounds are accumulated by the cell in the way in which Cohen & Rickenberg (1955) have shown for the accumulation of β-galactosides by *Escherichia coli*. It is difficult to demonstrate the accumulation of a compound which is an essential metabolite and is rapidly oxidized, and for which suitable analogues are not readily available, but it is hoped to investigate this aspect at a later date.

Cohen & Monod (1957) have stated that the permeases are highly stereospecific and gave evidence for eight separate and distinct permeases for *Escherichia coli*. Shilo & Stanier (1957) found that tartrate-oxidizing pseudomonads had separate induced transport systems for each of the three tartrate isomers. If we assume that our experiments with *Pseudomonas aeruginosa* provide reasonable evidence for the existence of permeases for the acids of the tricarboxylic cycle, then an examination of the patterns of oxidative activity, i.e. the presence or absence of lag periods for organisms grown on different substrates, should give some indication of the possible number of specific permeases. From the results given in Table 1 it can be seen that growth on different substrates produced organisms with distinct adaptive patterns for the oxidation of citrate, acetate, fumarate and succinate, suggesting that there are separate permeases for each of these acids. With this organism malate, pyruvate and α-oxoglutarate always gave the same pattern whatever the growth medium, so that it is not possible to say whether or not they are carried into the cell by different permeases. An examination of the results which Barrett & Kallio (1953) found with *P. fluorescens* shows separate and distinct patterns for citrate, acetate, α-oxoglutarate, fumarate and succinate, but the patterns for malate and pyruvate oxidation were identical. The results given by Kogut & Podoski (1953) show separate and distinct patterns for citrate, acetate, malate, pyruvate and α-oxoglutarate, but do not distinguish between succinate and fumarate. If it be permissible to combine these results with three different pseudomonads, then all the substances we have discussed have distinct permeases. However, further work will be needed to establish conclusively the specificity of the permeases concerned with the transport of these organic acids.

The implicit assumption has been made that permease formation can be adaptive and is then induced by the presence of the relevant compound. Rickenberg *et al.* (1956) showed that β-galactoside-permease is induced by compounds which are also inducers of the enzyme β-galactosidase, but that the capacity of different compounds to induce the permease is not always the same as their capacity to induce the enzyme β-galactosidase. In the experiments which we have described with *Pseudomonas aeruginosa* we have interpreted the results as showing that the substrates of the oxidative enzymes induce the formation of their own permeases, and that whereas the enzymes concerned in the tricarboxylic acid cycles are constitutive, the permeases are adaptive. It has been suggested that if the substrate of an adaptive enzyme were an essential cell-metabolite then, since it would always be present in the cell, it would induce its enzyme which would then appear as a constitutive enzyme. In the same way one might expect that a compound which was a normal cell-metabolite would be able to induce the formation of its permease. In the case of the substrates which we have investigated, which are all normal cell-metabolites, it may be that the concentration of the free compound in the cell when it has not been added to the medium, is too low to induce the permease. It might also be possible that permease production is suppressed, so that compounds which are essential metabolites are retained in the cell. There is evidence in certain organisms that permease production may be inhibited by other compounds present in the growth medium. Rickenberg *et al.* (1956) showed that the induction of β-galactoside-permease, as well as galactosidase, was inhibited by glucose. H. Green, reported by Davis (1956), found that *Aerobacter aerogenes* grown on glucose, but not on succinate, was impermeable to citrate. When organisms were grown on citrate with glucose, citrate utilization was inhibited, although extracts of the organisms still oxidized citrate. In these experiments glucose appeared to inhibit the formation of citrate-permease.

The site of the bacterial permeases has not yet been finally established, although all the available evidence would support the hypothesis that they are situated in the protoplast membrane of Gram-positive bacteria and in whatever is the equivalent osmotic barrier in Gram-negative bacteria. Rickenberg (1957) showed that the osmotically-fragile bodies formed by lysozyme treatment of *Escherichia coli* retained the ability of whole organisms for accumulating β-galactosides. The permeases must therefore be associated with those layers of the bacterial cell wall which are unchanged by lysozyme treatment. If the action of chloramphenicol towards Gram-positive and Gram-negative bacteria is similar, then our work showing that chloramphenicol inhibits permease synthesis would suggest that the permeases are not situated in the rigid layers of the wall. Mandelstam & Rogers (1958) and Hancock & Park (1958) showed that in *Staphylococcus aureus* chloramphenicol inhibited protein synthesis but not the synthesis of cell walls. Moreover, Crathorn & Hunter (1958) found that although chloramphenicol did not inhibit the synthesis of the cell-wall peptide, which appears to be elaborated in the protoplast membrane of *Bacillus megaterium*, it inhibited the incorporation of amino acids into the protein (Gilby, Few & McQuillen, 1958) present in the membrane itself. Evidence of the site of citrate-permease in *Aerobacter aerogenes* has been provided by some experiments by Green, reported by Davis (1956). He showed that the barrier to citrate-permeability in glucose-grown organisms was situated in the outer layers of the organism rather than at sites within it.

The pseudomonads are an omnivorous group of organisms which are capable of using a very large number of organic compounds of carbon and nitrogen. If each of these substances required a separate permease and enzyme, the problem of accommodating all these protein molecules becomes considerable, particularly as many of the enzymes, as well as the permeases, are probably associated with the osmotic barrier of the cell or at least with the 'small particle' fraction (Alexander, 1956). It seems likely that the adaptive formation of many of the enzymes and permeases has provided the organisms with a means of overcoming this difficulty.

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REFERENCES

ALEXANDER, M. (1956). Localization of enzymes in the microbial cell. *Bact. Rev.* **20**, 67.

BARRETT, J. T. & KALLIO, R. E. (1953). Terminal respiration in *Pseudomonas fluorescens*. Component enzymes of the tricarboxylic acid cycle. *J. Bact.* **66**, 517.

COHEN, G. N. & MONOD, J. (1957). Bacterial permeases. *Bact. Rev.* **21**, 169.

COHEN, G. N. & RICKENBERG, H. V. (1955). Étude directe de la fixation d'un inducteur de la β-galactosidase par les cellules d'*Escherichia coli*. *C.R. Acad. Sci., Paris*, **240**, 466.

CRATHORN, A. R. & HUNTER, G. D. (1958). Amino acid 'exchange' and protein synthesis in cell walls of *Bacillus megaterium*. *Biochem. J.* **69**, 47 P.

CREASER, E. H. (1956). The assimilation of amino acids by bacteria. 22. The effect of 8-azaguanine upon enzyme formation in *Staphylococcus aureus*. *Biochem. J.* **64**, 539.

Davis, B. D. (1956). Relations between enzymes and permeability (membrane transport) in bacteria. In *Enzymes: Units of Biological structure and Function. Henry Ford Hosp. Symp.* **4**, 509.

Gilby, A. R., Few, A. V. & McQuillen, K. (1958). The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus*. *Biochim. biophys. Acta*, **29**, 21.

Gray, C. T. (1952). The malonic decarboxylase of *Pseudomonas aeruginosa*. *J. Bact.* **63**, 813.

Hancock, R. & Park, J. T. (1958). Cell-wall synthesis by *Staphylococcus aureus* in the presence of chloramphenicol. *Nature, Lond.* **181**, 1050.

Hayaishi, O. (1954). Mechanism of the enzymatic decarboxylation of malonic acid. *Fed. Proc.* **13**, 226.

Kogut, M. & Podoski, E. P. (1953). Oxidative pathways in a fluorescent *Pseudomonas*. *Biochem. J.* **55**, 800.

Mandelstam, J. & Rogers, H. J. (1958). Chloramphenicol-resistant incorporation of amino-acids into staphylococci and cell-wall synthesis. *Nature, Lond.* **181**, 956.

Meadow, P. & Clarke, P. H. (1958). The oxidation of tricarboxylic acid cycle intermediates by *Pseudomonas aeruginosa*. *Biochem. J.* **69**, 18 p.

Milner, H. W., Lawrence, N. S. & French, C. S. (1950). Colloidal dispersion of chloroplast material. *Science*, **111**, 633.

Rickenberg, H. V. (1957). The site of galactoside-permease activity in *Escherichia coli*. *Biochim. biophys. Acta*, **25**, 206.

Rickenberg, H. V., Cohen, G. N., Buttin, G. & Monod, J. (1956). La galactoside-permease d'*Escherichia coli*. *Ann. Inst. Pasteur*, **91**, 829.

Shilo, M. & Stanier, R. Y. (1957). The utilization of tartaric acids by Pseudomonads. *J. gen. Microbiol.* **16**, 482.

Stone, R. W. & Wilson, P. W. (1952). Respiratory activity of cell-free extracts from azotobacter. *J. Bact.* **63**, 605.

Wolfe, J. B., Ivler, D. & Rittenberg, S. C. (1954). Malonate decarboxylation by *Pseudomonas fluorescens*. 1. Observations with dry cells and cell-free preparations. *J. biol. Chem.* **209**, 867.

Wolfe, J. B., Ivler, D. & Rittenberg, S. C. (1955). Malonate oxidation by dry cells and cell-free extracts of *Pseudomonas aeruginosa*. *J. Bact.* **69**, 240.

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Clarke, P. H., Glover, P. & Mathias, A. P. (1959). *J. gen. Microbiol.* **20**, 156–164

The Occurrence of Polyol Derivatives of Cytidine Diphosphate in Micro-organisms

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SUMMARY: A survey of cytidine diphosphate compounds in representative species of Gram-positive and Gram-negative bacteria has been made. Cytidine diphosphate glycerol and cytidine diphosphate ribitol, first discovered in *Lactobacillus arabinosus* 17/5, were also found in *L. plantarum.* Cytidine diphosphate glycerol was found in *L. bulgaricus* and cytidine diphosphate ribitol in *Bacillus subtilis.* No correlation between the presence of cytidine nucleotides and the Gram reaction was found.

Two derivatives of cytidine diphosphate were found in extracts of heat-killed *Lactobacillus arabinosus* 17/5 (Baddiley & Mathias, 1954) and were shown to be cytidine diphosphate glycerol (CDPglycerol; Baddiley, Buchanan, Mathias & Sanderson, 1956) and cytidine diphosphate ribitol (CDPribitol; Baddiley, Buchanan, Carss & Mathias, 1956). The α-glycerophosphate moiety of CDPglycerol has the L-configuration (Baddiley, Buchanan & Carss, 1956) in common with the α-glycerophosphate isolated from other natural sources (Baer & Fischer, 1939). By enzymic methods it has been shown that CDPribitol contains a D-ribitol-5-phosphate residue, which probably would be formed by the reduction of D-ribose-5-phosphate. Two other cytidine diphosphate derivatives are known: cytidine diphosphate choline and cytidine diphosphate ethanolamine. These are involved in the incorporation of phosphoryl choline or phosphoryl ethanolamine into $\alpha\beta$-diglycerides with the formation of lecithins or cephalins (Kennedy & Weiss, 1956). It is not yet known what roles CDPglycerol and CDPribitol play in the metabolism of glycerol and ribitol.

Mitchell & Moyle (1951*a*, *b*) isolated a glycerophosphate compound, mainly occurring in the cell envelope and small particle fractions of *Staphylococcus aureus* (*Micrococcus pyogenes*). No evidence was presented for the structure of this material, but the ease of hydrolysis in alkali fits in well with a linear polymer composed of glycerol residues linked 1:2 or 1:1 through a phosphodiester group. Mitchell & Moyle (1954) examined a number of Gram-positive and Gram-negative organisms, and reported the presence of other unidentified polyol phosphates in Gram-positive organisms and suggested that a lipid-polyol phosphate-protein component might be a common structural feature of Gram-positive bacteria. If one of the unidentified polyol phosphates were ribitol phosphate, the cells might contain a polyribitol phosphate, or a material in which either ribitol or glycerol residues were joined through a phosphodiester link. If such compounds did indeed exist in bacteria, then it would be reasonable to suppose that CDPribitol and CDPglycerol were involved in their biosynthesis. Furthermore, if the suggestion of Mitchell & Moyle (1954) were right, one might expect to find CDPribitol and CDPglycerol in extracts of Gram-positive organisms, but not in Gram-negative organisms. Accordingly, a survey was made to determine the distribution of the cytidine diphosphate compounds. The organisms investigated included most of those used by Mitchell & Moyle (1954) and a number of lactobacilli.

METHODS

Cultures and growth conditions. Lactobacillus arabinosus 17/5 was obtained from Dr D. E. Hughes, *L. plantarum* NCIB 8030 from the National Collection of Industrial Bacteria, and the other cultures from the National Collection of Type Cultures (NCTC). *Streptococcus lactis* H 240 came originally from the collection of Dr P. M. F. Shattock.

Lactobacillus arabinosus 17/5 was grown under the conditions described by Baddiley & Mathias (1954) in 5 l. batches. *L. bulgaricus* NCTC 76 and *L. plantarum* NCIB 8030 were subcultured from milk into 100 ml. flasks containing 50 ml. tomato juice broth (Briggs, 1953) and these were used to inoculate four flasks, each containing 2·5 l. of the same medium, which were incubated at 37° for 2 days.

Escherichia coli NCTC 86, *Klebsiella aerogenes* NCTC 418 and *Bacillus cereus* NCTC 945 were grown in 1 % (w/v) glucose broth containing (%, w/v): Lab. Lemco, 1; peptone, 1; sodium chloride, 0·5; glucose, 1; made up in distilled water. Each batch consisted of 8 l. of medium distributed as 2 l. in each of four 5 l. Pyrex bottles, which were aerated by continuous rotation on an electrically-driven mechanical rotator for 18–24 hr. at about 27°.

Streptococcus lactis H 240 and *Staphylococcus aureus* NCTC 4163 were grown under the same conditions as *Escherichia coli* with the medium supplemented by the addition of 25 ml. of a 10 % (w/v) yeast extract/l. medium. For the experiment on the effect of penicillin on nucleotide accumulation, *Staphylococcus aureus* NCTC 4163 was grown for 12 hr. in 4 l. of medium as above, the cocci were harvested by centrifugation in an MSE refrigerated centrifuge at 1500 *g* for 30 min. and resuspended in 2 l. of fresh broth containing 10 units penicillin/ml. and incubated for a further 3 hr.

Bacillus subtilis NCTC 3610 was inoculated from a Lemco agar slope culture into 20 tubes, each containing approximately 10 ml. of 1 % (w/v) glucose broth, which were incubated with shaking for 8 hr. at 37°. The broth cultures were used to inoculate 40 Roux bottles each containing 100 ml. Lemco agar. The Roux bottles were incubated 18 hr. at 37° and the organisms washed off with *c.* 1 l. distilled water, filtered through gauze and centrifuged for 15 min. in an MSE Angle 13 centrifuge at 4000 *g*.

Preparation of the nucleotide extracts from the bacteria. The organisms, grown in liquid culture, were harvested in a Sharples centrifuge, weighed and immediately extracted with aqueous ethanol (65 %, v/v), allowing 30 ml./10 g. wet organisms. The mixture was heated rapidly to the boiling point with vigorous stirring, boiled for 5 min., cooled and centrifuged. The precipitate was extracted with a similar volume of boiling aqueous ethanol (50 %, v/v).

The combined extracts were brought to pH 5 with dilute nitric acid and a slight excess of a solution of mercuric acetate in acetic acid added (Caputto, Leloir, Cardini & Paladini, 1950). After 16 hr. at 0°, the precipitate was centrifuged down and washed with a little ethanol (50 %, v/v). It was suspended in water and a vigorous stream of hydrogen sulphide passed through the suspension to decompose the mercury salts. The mercury sulphide was removed by centrifugation, the supernatant fluid aerated to remove hydrogen sulphide, adjusted to pH 8 with ammonia, and freeze-dried.

Ion-exchange chromatography. A column ($1{\cdot}5 \times 17{\cdot}5$ cm.) of Dowex-1 (formate-form; 200–400 mesh) was prepared according to the method of Hurlbert, Schmitz, Brumm & Potter (1954). A solution of the nucleotide ammonium salts (0·2–0·7 g.) in water (25–50 ml.), adjusted to pH 8·5, was added to the column at a rate of 0·3 ml./min. The column was then washed with water until the absorption of the eluate at 260 mμ had fallen to 0·1. Elution was effected by the use of a linear gradient of formic acid. This was obtained by employing two identical cylindrical vessels as the reservoir and the mixing flasks, which were connected by a syphon (Drake, 1955). The mixing flask was joined to the column by a short length of polythene tubing. Approximately 10 ml. of liquid were kept above the surface of the resin to prevent disturbance by splashing. The solution in the mixing vessel was stirred 'magnetically'. At the start, the mixing vessel contained 1 l. of water and the reservoir 1 l. of 5 N-formic acid. The flow was adjusted to 0·3–0·4 ml./min., and the eluate collected in 7·2 ml. fractions. The absorption at 260 mμ of each fraction was measured in a 1 cm. cell. To help in the identification of the base, the absorption at 275 mμ of the tubes corresponding to the maximum of each peak was also determined. The fractions containing each peak were combined and freeze-dried. At the end of each run, the column was regenerated by treatment with a solution of 6 N-formic acid containing 4 N-ammonium formate.

Paper chromatography of the nucleotide peaks. Each peak-eluate was examined by ascending chromatography on Whatman no. 4 paper, previously washed with 2 N-acetic acid followed by water. The solvents used were: *iso*butyric acid + ammonium *iso*butyrate + water (Magasanik *et al.* 1950); propanol + ammonia + water (Hanes & Isherwood, 1949); ethanol + ammonium acetate (Paladini & Leloir, 1952). Several samples of each eluate were run in each solvent together with the appropriate markers. The papers also had blank tracks. After chromatography and removal of the solvents, the chromatograms were examined under ultraviolet radiation and the absorbing spots marked with pencil. The paper was cut into strips. The u.v.-absorbing spots from one track were cut out, eluted with 3 ml. of 0·01 N-hydrochloric acid, and the u.v. absorption spectrum determined, using as a blank the eluate from a spot of similar area and R_F taken from a blank track. The eluates were then adjusted to pH 12 by the addition of 2 N-sodium hydroxide, and the spectrum redetermined. The other tracks were treated with one of the following sprays: the phosphate reagents of Hanes & Isherwood (1949); the periodate-Schiff reagent (Baddiley, Buchanan, Handschumacher & Prescott, 1956), and in the case of suspected nicotinamide derivatives, with the methylethylketone + ammonia reagent (Kodicek & Reddi, 1951). In the few cases in which the base could not be definitely identified from the u.v. absorption spectrum, another sample of the band under investigation was eluted from the paper, evaporated to dryness and hydrolysed with perchloric acid (72 %, w/v) according to the method of Marshak & Vogel (1951). The hydrolysate was run on paper with the *iso*propanol + hydrochloric acid system (Wyatt, 1951), and the purine or pyrimidine base identified by comparison of its R_F value with that of authentic specimens and from its u.v. absorption spectrum.

The cytidine compounds from peaks C and D were readily distinguishable on paper in the *iso*butyric acid solvent, having R_F values of 0·15 and 0·22, respectively. Both reacted rapidly with the periodate spray. To confirm the structures assigned to them, samples were eluted from paper chromatograms with a few drops of N-hydrochloric acid, and the eluate heated in a sealed tube at 100° for 2–3 hr. After evaporation of the acid, the hydrolysate was separated chromatographically with the propanol + ammonia solvent. The products were detected by examination under u.v. radiation and by spraying with the phosphate and the periodate-Schiff reagents. CDPribitol gave CMP and mainly anhydro-ribitol. CDPglycerol gave CMP and glycerophosphate.

RESULTS

The same column of ion-exchange resin was used throughout these experiments and was standardized by an examination of the nucleotides from heat-killed *Lactobacillus arabinosus* 17/5, which have already been investigated (Baddiley, Buchanan, Carss, Mathias & Sanderson, 1956). The elution pattern obtained from *L. arabinosus* is shown in Fig. 1. The other organisms gave similar patterns, with the exception of peaks D and E, and there would seem to be no gross differences amongst them in the amounts of DPN, AMP and ADP present.

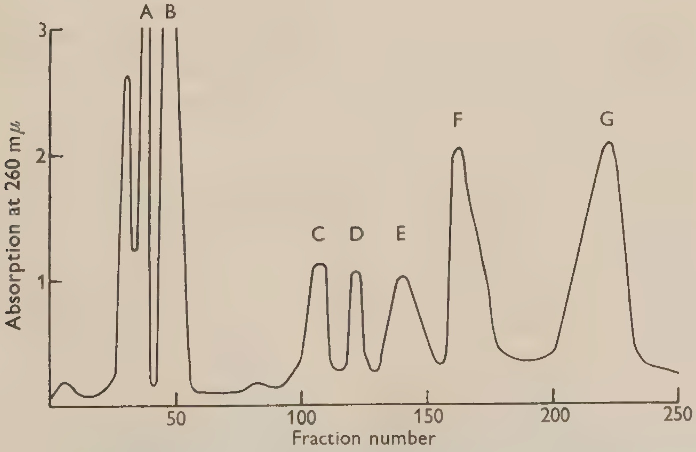

Fig. 1. Diagram of the elution of nucleotides of *Lactobacillus arabinosus* from Dowex-1. The lettering of the peaks refers to Table 1.

The nucleotides found in the organisms in this survey are summarized in Table 1. Elution was discontinued after the strength of the eluant had almost reached 4N-formic acid, by which time adenosine diphosphate had been displaced from the column. As will be seen from Table 1, the cytidine diphosphate derivatives were limited in their distribution. CDPribitol and CDPglycerol were found in *Lactobacillus arabinosus* and *L. plantarum*. However, *L. bulgaricus* contained only CDPglycerol, while *Bacillus subtilis* contained only CDPribitol. In *B. subtilis* the amount of CDPribitol found, relative to the AMP peak, was only about a fifth of that in *L. arabinosus* or *L. plantarum*. The only other organism in which these cytidine compounds were detected was *Staphylococcus aureus* under conditions of penicillin stasis. A small peak possessing a cytidine spectrum emerged from the column in the position normally occupied by CDPribitol, but the amounts present were too small for an investigation by paper chromatography.

DISCUSSION

It is now known that there are marked differences in the chemical composition of the cell walls of Gram-positive and Gram-negative bacteria with respect to lipid, amino acid and carbohydrate content (Salton, 1953; Cummins & Harris, 1956; Work 1957). Mitchell & Moyle (1954) determined the phosphorus-containing compounds of a number of Gram-positive and Gram-negative organisms after lipid extraction, and concluded that the Gram-positive organisms contained a phosphoric ester fraction (XSP) which was absent from the Gram-negative organisms. They had previously found that in *Staphylococcus aureus* (*Micrococcus pyogenes*) at least part of this XSP appeared to be a polyglycerophosphate compound. Jones, Ritzvi & Stacey (1958), using a more detailed method of analysis, presented evidence that this non-nucleotide phosphorus fraction occurred in preparations made from whole organisms of both Gram-positive and Gram-negative organisms. They identified two components from the hydrolysate of the XSP fraction from *Klebsiella pneumoniae* as glycerophosphate and inositol phosphate. It should be noted, however, that the analyses of Jones *et al.* (1958) show no correlation between the Gram-staining reaction and the content of ribonucleic acid or the ratio of ribonucleic acid and deoxyribonucleic acid.

The discovery that ribitol phosphate is present in a combined form in large amounts in the cell walls of *Lactobacillus arabinosus*, *Bacillus subtilis* and *Staphylococcus aureus* (Baddiley, Buchanan & Carss, 1958), provided an explanation for some part of the XSP fraction. Further studies have shown that the ribitol polymer from *L. arabinosus* also contains glucose and alanine (Armstrong, Baddiley, Buchanan & Carss, 1958). No glycerol polymer was found in the cell-wall fraction but it was suggested that it might be a component of the cytoplasmic membrane. It is only possible to distinguish clearly between the cell wall and the cytoplasmic membrane in Gram-positive bacteria where the cell wall can be stripped off by agents such as lysozyme, leaving protoplasts. Some information on the composition of the cytoplasmic membrane

Table 1. *Distribution of cytidine diphosphate compounds in various micro-organisms*

Organism	Weight of wet cells (g.)	Weight of nucleotide fraction (g.)	Gram reaction	Peak A	Peak B	Peak C	Peak D	Peak E	Peak F	Peak G
Lactobacillus arabinosus 17/5	16·5	0·3	+	CMP*+DPN	AMP†	CDPribitol	CDPglycerol	Mainly GMP	UMP IMP	ADP
L. plantarum NCIB 8030	17·0	0·3	+	CMP*+DPN	AMP	CDPribitol	CDPglycerol	Mainly GMP	UMP	ADP
L. bulgaricus NCTC 76	17·0	0·4	+	CMP*+DPN	AMP	0	CDPglycerol	GMP	UMP	ADP
Escherichia coli NCTC 86	20·0	0·3	−	DPN, trace CMP	AMP	0	0	GMP	UMP	ADP
Bacillus cereus NCTC 945	16·0	0·4	+	DPN, trace CMP	AMP	0	0	GMP	UMP	ADP
Klebsiella aerogenes NCTC 418	35	0·7	−	DPN	AMP	0	0	GMP	UMP	ADP
Staphylococcus aureus NCTC 4163	20	0·6	+	DPN, CMP	AMP†	0	0	GMP	UMP	ADP
S. aureus + penicillin	8	0·2	(+)	DPN, CMP	AMP	v.s.	0	GMP	Mainly UMP	ADP
Streptococcus lactis H 240	24	0·4	+	DPN,* trace CMP	AMP	0	0	GMP	UMP	ADP
Bacillus subtilis NCTC 3610	31	0·4	+	DPN, CMP	AMP	CDPribitol	0	GMP	UMP IMP	ADP

* Preceded by an unidentified compound that gave a negative reaction for phosphorus and with the periodate-Schiff reagent. Absorption maximum at 248 mμ in acid and 259 in alkali, minimum at 225 in acid and 235 in alkali.

† Followed by a small adenine peak, possibly TPN.

The abbreviations used are: AMP, adenosine-5′-phosphate; ADP, adenosine diphosphate; CMP, cytidine-5′-phosphate; DPN, diphosphopyridine nucleotide; GMP guanosine-5′-phosphate; IMP, inosine-5′-phosphate; UMP, uridine-5′-phosphate. 0, no peak detected; v.s. very small amount of a cytidine compound.

of a Gram-positive organism is given by Weibull (1957) who examined the lipids of a lysozyme-sensitive bacillus and found that 65 % of the total lipids appeared to be located in the cytoskeletons remaining after the cells had been lysed with lysozyme. The lipids from this fraction appeared to consist of a mixture of neutral fats, fatty acids and phosphatidic acids. Gilby, Few & McQuillen (1958) examined the chemical composition of the protoplast membrane of *Micrococcus lysodeikticus* and found that the lipid component had a similar absence of nitrogen-containing compounds and appeared to consist of approximately 80 % of complex phosphatidic acids. The resistance to hydrolysis by methanolic potassium hydroxide suggested that these were not simple phosphatidic acids. The lipid fraction contained about 73 % of the phosphorus present in the intact membrane, thus leaving 27 % to be accounted for in the non-lipid fraction.

If a ribitol phosphate polymer is a normal cell-wall component, and a glycerophosphate polymer a normal cytoplasmic membrane component, it would seem likely that cytidine diphosphate ribitol and cytidine diphosphate glycerol are involved in their biosynthesis. If this were so, it might have been expected that the cytidine diphosphate derivatives would have been of more general distribution than this survey suggests. Failure to demonstrate these compounds may simply mean that their normal concentration within the cell is too low for detection by these methods. The experiment in which *Staphylococcus aureus* was treated with penicillin showed that a small amount of cytidine compound accumulated under conditions similar to those in which Park (1952) found accumulation of the uridine nucleotides which are thought to be cell-wall precursors (Park & Strominger, 1957). If the glycerol and ribitol polymers are found to be of widespread occurrence in bacteria, then the exceptional organisms may be those which accumulate the cytidine nucleotides under normal growth conditions. Of the two *Bacillus* species examined, *Bacillus subtilis*, which has been shown by Baddiley *et al.* (1958) to contain a ribitol phosphate polymer in the cell wall, was found by us to contain considerable amounts of cytidine diphosphate ribitol. This was not found in the extracts of *B. cereus* and it would be interesting to know whether the cell wall of this species contains a ribitol phosphate polymer.

The distribution of the cytidine diphosphates among the lactobacilli examined fits in well with current ideas on their classification. It is not surprising that *Lactobacillus plantarum* NCIB 8030 should resemble *L. arabinosus* 17/5. Briggs (1953) classed *L. arabinosus* with *L. plantarum* and *L. casei* in group VI of her classification of lactobacilli based on physiological tests. Wheater (1955*b*) also included *L. arabinosus* with *L. plantarum*. Sharpe (1955) in a serological study of lactobacilli found that four strains described as *L. arabinosus* fell into the *L. plantarum* serological group. *Lactobacillus bulgaricus* on the other hand can be distinguished from *L. arabinosus* and *L. plantarum* by physiological tests (Briggs, 1953, Wheater, 1955*a*), and serological reactions (Sharpe, 1955). Since only cytidine diphosphate glycerol, and not cytidine diphosphate ribitol, was found in the extracts from *L. bulgaricus* it would be interesting to know whether or not the ribitol polymer will be found in the cell wall. The species distribution of these polyol phosphate polymers in bacterial structures may turn out to be fairly wide, if they can be equated with all or part of the XSP fractions described by Mitchell & Moyle (1954) and Jones *et al.* (1958). If this should prove to be the case, it would be interesting to know the factors which cause the accumulation of relatively large amounts of the cytidine nucleotides, which may be precursors, in the species we have described. In considering the possible functions of CDPribitol and CDPglycerol, it is worth noting that in the biosynthetic reactions of both the other CDP compounds, CDPcholine and CDPethanolamine, cleavage of the pyrophosphate link occurs. Thus if these compounds are represented by the general formula CDPX, then it is PX that is transferred. It might be argued that in the case of the polyol derivatives of cytidine diphosphate, one would expect to find that it was the polyol phosphate that was incorporated as a unit in any polymerization reactions. This is in contrast to the reactions of the sugar derivatives of uridine diphosphate (UDP). In the transferase systems in which these take part, the sugar is transferred without a phosphate ester group and UDP is formed.

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REFERENCES

Armstrong, J. J., Baddiley, J., Buchanan, J. G. & Carss, B. (1958). Nucleotides and the bacterial cell wall. *Nature, Lond.* **181**, 1692.

Baddiley, J., Buchanan, J. G. & Carss, B. (1956). The configuration of the ribitol phosphate residue in cytidine diphosphate ribitol. *J. chem. Soc.* p. 1869.

Baddiley, J., Buchanan, J. G. & Carss, B. (1958). The presence of ribitol phosphate in bacterial cell walls. *Biochim. biophys. Acta*, **27**, 220.

Baddiley, J., Buchanan, J. G., Carss, B. & Mathias, A. P. (1956). Cytidine diphosphate ribitol from *Lactobacillus arabinosus*. *J. chem. Soc.* p. 4583.

Baddiley, J., Buchanan, J. G., Carss, B., Mathias, A. P. & Sanderson, A. R. (1956). The isolation of CDPglycerol, CDPribitol and Manitol 1-Phosphate from *Lactobacillus arabinosus*. *Biochem. J.* **64**, 599.

Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). Chemical studies in the biosynthesis of purine nucleotides. Part I. The preparation of *N*-glycylglycosylamines. *J. chem. Soc.* p. 2818.

Baddiley, J., Buchanan, J. G., Mathias, A. P. & Sanderson, A. R. (1956). Cytidine diphosphate glycerol. *J. chem. Soc.* p. 4186.

Baddiley, J. & Mathias, A. P. (1954). Cytidine nucleotides. Part I. Isolation from *Lactobacillus arabinosus*. *J. chem. Soc.* p. 2723.

Baer, E. & Fischer, H. O. L. (1939). Studies on Acetone-Glyceraldehyde. VI. Synthesis of the biological L(–) α-glycerophosphoric acid. *J. biol. Chem.* **128**, 491.

Briggs, M. (1953). The classification of lactobacilli by means of physiological tests. *J. gen. Microbiol.* **9**, 234.

Caputto, R., Leloir, L. F., Cardini, C. E. & Paladini, A. C. (1950). Isolation of the coenzyme of the galactose phosphate-glucose phosphate transformation. *J. biol. Chem.* **184**, 333.

Cummins, C. S. & Harris, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.

Drake, B. (1955). Contributions to the theory of gradient elution analysis. *Ark. Kemi*, **8**, 1.

GILBY, A. R., FEW, A. V. & MCQUILLEN, K. (1958). The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus*. *Biochim. biophys. Acta*, **29**, 21.

HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107.

HURLBERT, R. B., SCHMITZ, H., BRUMM, A. F. & POTTER, V. R. (1954). Nucleotide metabolism. II. Chromatographic separation of acid soluble nucleotides. *J. biol. Chem.* **209**, 23.

JONES, A. S., RITZVI, S. B. H. & STACEY, M. (1958). The phosphorus-containing compounds of Gram-positive and Gram-negative organisms in relation to the Gram staining reaction. *J. gen. Microbiol.* **18**, 597.

KENNEDY, E. P. & WEISS, S. B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. biol. Chem.* **222**, 193.

KODICEK, E. & REDDI, K. K. (1951). Paper chromatography of nicotinic acid derivatives. *Nature, Lond.* **168**, 475.

MAGASANIK, B., VISCHER, E., DONIGER, R., ELSON, D. & CHARGAFF, E. (1950). The separation and estimation of ribonucleotides in minute quantities. *J. biol. Chem.* **186**, 37.

MARSHAK, A. & VOGEL, H. J. (1951). Microdetermination of purines and pyrimidines in biological materials. *J. biol. Chem.* **189**, 597.

MITCHELL, P. & MOYLE, J. (1951*a*). Isolation of hydrolytic products of a glycerophospho-compound from *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 966.

MITCHELL, P. & MOYLE, J. (1951*b*). The glycerophospho-protein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

MITCHELL, P. & MOYLE, J. (1954). The Gram reaction and cell composition: Nucleic acids and other phosphate fractions. *J. gen. Microbiol.* **10**, 533.

PALADINI, A. C. & LELOIR, L. F. (1952). Studies on uridine-diphosphate-glucose. *Biochem. J.* **51**, 426.

PARK, J. T. (1952). Uridine-5′-pyrophosphate derivatives. Isolation from *Staphylococcus aureus*. *J. biol. Chem.* **194**, 877.

PARK, J. T. & STROMINGER, J. L. (1957). Mode of action of penicillin. *Science*, **125**, 99.

SALTON, M. R. J. (1953). The composition of the cell walls of some Gram positive and Gram-negative bacteria. *Biochim. biophys. Acta*, **10**, 512.

SHARPE, M. E. (1955). A serological classification of lactobacilli. *J. gen. Microbiol.* **12**, 107.

WEIBULL, C. (1957). The lipids of a lysozyme sensitive Bacillus species. (Bacillus 'M'). *Acta chem. Scand.* **11**, 881.

WHEATER, D. M. (1955*a*). The characteristics of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. *J. gen. Microbiol.* **12**, 123.

WHEATER, D. M. (1955*b*). The characteristics of *Lactobacillus plantarum, L. helveticus* and *L. casei*. *J. gen. Microbiol.* **12**, 133.

WORK, E. (1957). Biochemistry of the bacterial cell wall. *Nature, Lond.* **179**, 841.

WYATT, G. R. (1951). The purine and pyrimidine composition of deoxypentose nucleic acids. *Biochem. J.* **48**, 584.

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Wiame, J. M., Harpigny, R. & Dothey, R. G. (1959). *J. gen. Microbiol.* **20**, 165–172.

A New Type of Acetobacter: *Acetobacter acidophilum* prov. sp.

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SUMMARY: A new type of Acetobacter, provisionally named *Acetobacter acidophilum*, has been isolated in pure culture from an experimental vinegar generator originally inoculated with active industrial beechwood shavings. In contrast to other bacteria, this organism needs an acidic medium for growth and has a very specific oxidizing capacity. It does not grow above pH 4·3 and oxidizes only primary alcohols. Other substrates, including glucose and acetic acid, are not oxidized at a rate higher than 1 % of the oxidation rate for ethanol. The new isolation procedure, which has been applied for the strain described in this paper, allows the isolation of the bacteria present on active shavings, suggesting that this type of bacteria belongs probably to the group of organisms responsible for the acetification in the 'quick vinegar process'.

The microbiology of the industrial production of vinegar by the most common procedure, the so-called 'quick vinegar process', is still very obscure. Although not very often explicitly stated, the difficulty of isolating bacteria from the beechwood shavings, as well as from the liquid of the vinegar generator, is well known. This is the reason that prompted us to investigate this problem. These difficulties could hamper the solution of an interesting problem of bacterial ecology, which is of some practical importance.

The bacteria called by Henneberg (1926) the 'Schnellessigbakterien' (*Acetobacter acetigenum*, *A. schützenbachi* and *A. curvum*) were believed by this author to be the organisms which are responsible for the process. The opinion of Henneberg has been repeated uncritically in some treatises on industrial microbiology. Recent works on Acetobacter have not included studies of these species. They are either neglected or relegated to mere listing in the appendix (*Bergey's Manual*, 1948, 1957). In Frateur's monograph (1950), the three species of Henneberg are not included for lack of sufficient description and, as mentioned by Frateur, the bacteria of the quick process remained an enigma. These Acetobacter are not identical with the known species, since they do not grow on the classical solid media. It has been possible to isolate Acetobacter from beechwood shavings, but always after an enrichment. In different media, such as beer, mineral media, after 1 week or more of immersion to half height of a shaving, some growth occurs and bacteria may be isolated by plating on yeast water + ethanol + $CaCO_3$ agar (Frateur, 1950). This method is not free of the pitfalls of selection inherent in this sort of isolation. The isolated organisms represent only a small proportion of the initial bacteria. In any event, it would have been necessary to test the isolated bacteria on a sterile experimental quick vinegar apparatus (3rd Koch postulate). For these reasons, we have avoided enrichment cultures. All efforts at isolation in neutral media were unsuccessful. However, the use of solid media containing yeast extract + ethanol acidified to pH 4·5 with acetic acid resulted in the growth of extremely small colonies, almost invisible to the naked eye. The results were, however, very irregular and it was difficult to transfer the bacteria from plate to plate. With this material, and also with the method of purification by extreme dilution, we were able to inoculate an experimental acidifier loaded with woodwool and a sterile medium similar to the one used in industry (Wiame & Lambion, 1950; Lambion & Wiame, 1951). An acidity of 11 % (w/v) of acetic acid was obtained after 25 days. In spite of such results, the difficulty of obtaining colonies and their successful transfer remained the main obstacle for a rational study of the bacteria.

After many attempts to improve the growth on solid medium, we tried an acid medium (pH 3·0) solidified by an acid-resistant gel. The result was very striking: small but workable colonies were observed, allowing us to study more carefully the physiology of this organism which is very different from Acetobacter organisms studied hitherto. By the procedure described in this paper it has been possible to obtain colonies regularly by directly plating material from shavings. However, this paper pertains only to one strain, leaving for further study the comparison of new strains isolated directly from industrial apparatus. At least, the properties of this organism explain the difficulties encountered in previous work. It is hoped that their elucidation will help to resolve the enigma of this very old industrial process.

METHODS

Isolation of Acetobacter acidophilum *on solid medium*

Since the organism has an optimal pH of 3·0–3·2 and grows slowly, it is difficult to solidify the medium with agar. The silica gel method of Winogradsky was used with success and gave visible colonies (Pl. 1, fig. 1). This method is cumbersome and, for this reason, we tried a new silica gel method proposed by Kingsbury & Barghoorn (1954). It consists in the use of a commercial colloidal suspension of silicic acid which, after mixture with the nutrient medium, is solidified by autoclaving. For the present purpose, we proceeded as follows.

The commercial colloidal silica product (Ludox, Dupont de Nemours and Co., Inc., Wilmington, Delaware, U.S.A.) was de-ionized on the two resins Amberlite IR 120 and Amberlite IR 45. The Amberlite IR 120 was used in the acid form and, when necessary, regenerated by passing two volumes of M-HCl. The Amberlite IR 45 was used in the basic form and regenerated by two volumes of NaOH 4 % (w/v). The resins after regeneration and washing with distilled water were always dried by suction in order to keep the product undiluted by the de-ionization procedure. The Ludox had an initial pH value of 10. This product was mixed with one volume of Amberlite IR 120; by filtration the Ludox was obtained at *c.* pH 2 and was mixed with one volume of Amberlite IR 45; after filtration, the pH value was *c.* 4·5. This product was used for the next step. One volume of each resin may be used for two or three volumes of Ludox, before regeneration.

The de-ionized Ludox was mixed with the liquid nutrient, in the proportion of 6·25 to 3·75 (v/v). After mixing, the final concentrations of ingredients of the medium were: yeast extract, 0·5 % (w/v), acetic acid, 3 % (w/v), ethanol, 4 % (v/v) and minerals (see liquid medium); the pH value was 3·2.

The mixture was poured into Petri dishes which were placed horizontally in the autoclave. The temperature was raised slowly; after 15 min. at 110°, the temperature was brought down slowly again. When the gel was well formed it was easy to use a thick platinum wire for inoculation. It was necessary to begin by the addition of a few drops of sterile water to the suspension of bacteria; dry plates at the start in general failed to grow. The plates were kept in a container which received a short flush of CO_2 (see later, requirement for CO_2). To avoid the formation of cracks in the gel after drying, it was best, after a few days, to seal the plates with paraffin. Slopes of silica gel can be made similarly. The bacteria remained viable for a long time under these conditions. The colonies grew slowly to a diameter of 0·5 mm., becoming denser and slightly brown. Illustrations of young colonies are given in Pl. 1, figs. 2, 3. The edge of a colony is shown in Pl. 1, fig. 4.

Growth in liquid medium

The following medium was used routinely: ethanol, 4 % (v/v); glacial acetic acid, 3 % (w/v); yeast extract (Difco), 0·5 % (w/v); $(NH_4)_2SO_4$, 0·002 M; K_2SO_4, 0·002 M; KH_2PO_4, 0·002 M; $MgSO_4$, 0·0002 M; ferric citrate, $2 \cdot 10^{-6}$ M. The ethanol was added after sterilization. The cultures were incubated at 30° in 40 ml. volumes of this medium dispensed in conical flasks of 125 ml. capacity. The cultures were aerated on a rotating shaker (120–140 rev./min.). After the sterilization of such an acid medium, it was found necessary to introduce a small quantity of CO_2 into the gas-phase to help start the growth. To keep the cultures in the active growing phase, 4 ml. culture were transferred every 48 hr. After 48 hr. the cultures reached an opacity of about 0·2–0·3 scale unit, when measured in a Spekker opacimeter, with 1 cm. light path. One ml. of a culture of optical density 1·0 corresponded to a dry weight of 0·25 mg.; 1 mg. dry weight corresponded to 0·11 mg./N. When the growth was followed, special flasks with a cylindrical side arm were used (Wiame & Storck, 1953). Aeration was obtained by using a magnetic stirrer. When a special gas-phase composition was needed, an injection of gas was done by a tube passing through the cottonwool plug.

RESULTS

Some characteristics of the organism

The *Acetobacter acidophilum* organisms are elongated, single, in pairs or at times in chains of four to five rods. Plate 2, fig. 5, shows the organism under phase contrast. The bacteria were not motile and electron microscopy did not show any evidence of flagella, even when the bacteria were grown directly on the films used for the preparation. Plate 2, figs. 6, 7, show the bacteria after growth in liquid medium. The electron micrograph revealed a rough appearance of the bacterial surface. The rods were unusually opaque to the electron beam. Treatment with ultrasonic waves for 15 min. (Raytheon 10 Kc.) broke the organisms into smaller pieces, but did not result in a marked decrease in opacity as occurs with other bacteria, e.g. *Bacillus subtilis* and *Escherichia coli*. The organisms in the active growing phase were Gram negative and the catalase test negative.

Effect of acidity and nutrients

Figures 1 and 2 show that the bacteria only grew at pH values 2·7 and 4·0. The best growth was obtained at pH 3·0–3·5 and no growth occurred above 4·3. This was true not only when the acidity was produced by acetic acid, but also with phosphoric acid.

The absolute quantity of acetic acid + acetate affected the growth. Growth was very limited when the molarity of the mixture was higher than 0·5 (Fig. 3). It is mainly with respect to this property that other strains should be studied.

As previously mentioned, the organism requires a complex medium, e.g. containing generally 0·5 % (w/v) yeast extract. With decreasing concentrations of yeast extract the growth decreased both in rate and total growth. The addition of guanine was slightly beneficial. All attempts to replace the yeast extract with defined mixtures have up to now failed; these included: the addition of folic acid, biotin, *p*-aminobenzoic acid, riboflavin, nicotinic acid, pyridoxine and pantothenic acid. Guanine, uracil and adenine alone, or in mixtures with other vitamins or casein hydrolysate, did not support growth.

Influence of CO_2

Upon sterilization of the acid medium the loss of CO_2 led to very irregular results. It was then observed that successful growth depended on the size of the neck of the flask, suggesting the need for the CO_2 of the atmosphere. This was tested by alternate depletion and addition of the CO_2 (Fig. 4). Depletion was done by passing the air through two gas-washers with NaOH, 10 % (w/v). The gas was then introduced into the atmosphere of the vessel through the cotton plug. The addition of CO_2 corresponded to *c.* 1 % (v/v) of the air inflow. The need for CO_2 in such a complex medium was unexpected, since the use of yeast extract has very often been shown to obviate the need for CO_2 (Lwoff, Audureau & Monod, 1947; Ajl & Werkman, 1948). In the present case, it seems that the need for CO_2 is not required for synthesis of amino acids derived from the tricarboxylic acids (Wiame & Bourgeois, 1955; Wiame, 1957), which are present in the yeast extract. Addition of all the members of the tricarboxylic acid cycle had no effect. Preliminary studies on the utilization of CO_2 show that it is not found mainly in glutamic or aspartic acids, but in compounds tentatively identified as the basic amino acids.

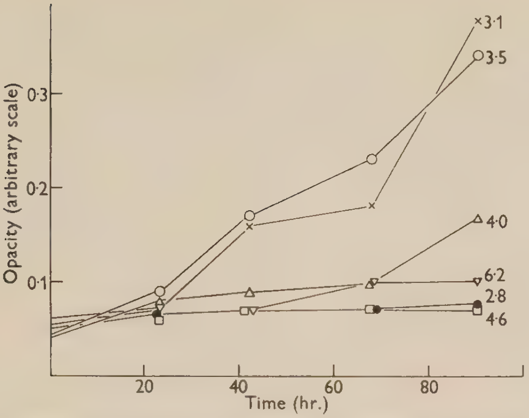


Fig. 1. Influence of acidity (acetic acid) on growth. Growth in liquid medium (see Methods) adjusted to different pH values, by NaOH (10%, w/v) or H_3PO_4, 0·04 M. Aeration by magnetic stirrer and continuous introduction of air. The numbers given on the curves indicate the initial pH values of the cultures.

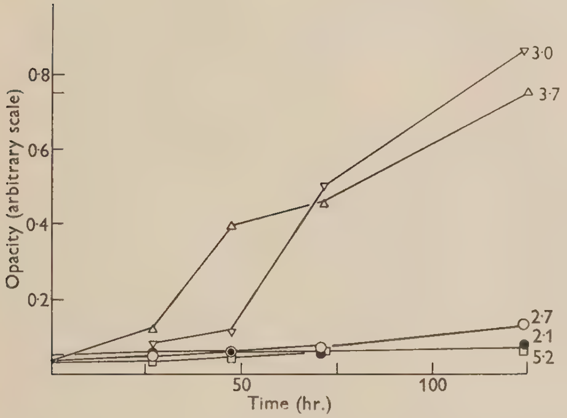


Fig. 2

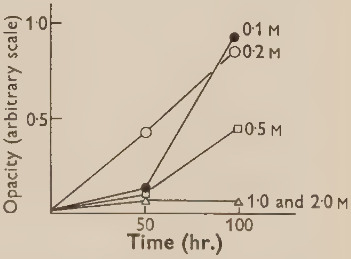


Fig. 3

Fig. 2. Influence of acidity (phosphoric acid) on growth. As in Fig. 1, but the acetic acid of the medium was replaced by mixtures of $KH_2PO_4+H_3PO_4$ (final concentration 0·3 M). The numbers given on the curves indicate the initial pH values of the cultures.

Fig. 3. Effect of the total quantity of acetic acid + acetate. The pH value was kept constant at 3·1. The total concentration of acetic acid + acetate was variable. Liquid medium, as previously, was used.

Oxidative capacities

All simple primary alcohols from C_2 to C_5 were oxidized at a considerable rate (Fig. 5). The oxidation stopped when an equivalent of acid was formed. Methanol was not oxidized. Isobutanol was not oxidized at the same rate as the normal alcohol. Among the secondary alcohols, isopropanol was slowly oxidized. Primary alcohols containing other groups, such as glycol and glycerol, were not oxidized.

With a suspension of organism which had an oxidative capacity of 4200 μl. O_2/hr./mg. N for ethanol, the value for the oxidation of glucose was only 17.

The following substances or mixtures were not oxidized at a measurable rate: acetic acid (40 and 10 μmole/vessel), lactic acid, pyruvic acid, α-keto glutaric acid, citric acid, succinic acid, malic acid (10 μmole/vessel). Endogenous respiration was not higher than the experimental error and there was no O_2 consumption with yeast extract alone or with acetic acid. The feeble oxidation of glucose was unexpected for an Acetobacter. Modification in pH value (3·1–6·5) did not change significantly the oxidation of glucose or acetic acid. *Acetobacter ascendens* (Henneberg) and *A. peroxydans* (Visser 't Hooft) are other types which do not oxidize glucose. All the tests reported here were done after growth on ethanol in the presence of acetic acid; it is not excluded that some substrates might be oxidized after suitable adaptation.

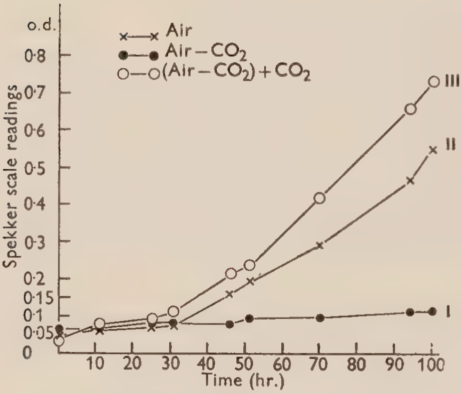


Fig. 4

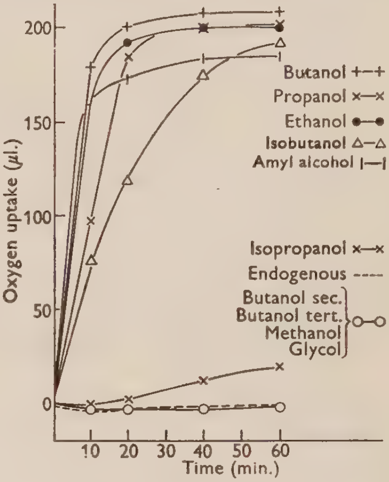


Fig. 5

Fig. 4. Effect of CO_2 on the growth of *Acetobacter acidophilum*. A freshly sterilized medium was used and the atmosphere kept constant by injections of gas mixtures. Curve I, air passed through NaOH; curve II, ordinary air; curve III, air, after being deprived of CO_2, with subsequent addition of pure CO_2 (*c*. 1 %).

Fig. 5. Oxidation of alcohols by *A. acidophilum* at 30°, tested by the standard manometric method, 10 μmole of all substrates. The main cup contained 2 ml. of a suspension of bacteria corresponding to 0·26 mg of bacterial nitrogen in phosphate buffer 0·04 M, pH 3·1. 0·2 ml. 5 N-NaOH was placed in the centre well. All substances were commercial products, analytical grade, without further purification. The break in the oxidation of amyl alcohol was probably due to the presence also of a branched-chain amyl alcohol.

The oxidation of ethanol, in contrast to growth, was not depressed at pH 5 or even at 6·5. In the acid range, at pH 3·1, 3 % (w/v) acetic acid depressed the rate of oxidation by about 50 %, and 7 % acetic acid decreased the oxidative capacity to 25 % of that obtained at pH 3·1 in phosphoric buffer (0·04 M).

DISCUSSION

From the results reported here it is easy to understand the difficulty which has been encountered in previous attempts to isolate bacteria such as *Acetobacter acidophilum*. The strain described in this work may be a 'domesticated' one, since it was isolated in an experimental apparatus, but, as said earlier, other strains may be isolated by direct plating and these are now under study. The striking properties of this organism permit the prediction that this type of Acetobacter is the active bacterium of the 'quick vinegar process'. Other strains may be more or less resistant to high concentrations of acetic acid. By using classical microbiological methods it should be easy to start new studies of many strains and to select those best adapted for industrial applications. The recent use of the submerged culture technique might also be improved in this way (Hromatka & Ebner, 1949, 1950).

It is interesting to note the unique character of our organism, in particular its acidophily as well as its very restricted oxidation ability. The organism most resistant to acid, *Thiobacillus thiooxidans*, can still grow at pH 6·0. *Acetobacter acidophilum* shows a type of physiology corresponding to a very specialized ecological habitat. If one tries to place this organism in the at present accepted classification of Acetobacter, one is confronted with the difficulty of using the present criteria, since these bacteria do not grow under the proposed conditions. However, our organism is a typical acetic acid bacterium, perhaps the most specialized one, since its best characteristic is the oxidation of ethanol to acetic acid and its growth obligately in an acidic medium. The lack of catalase might place it in the 'peroxydans' group, but this seems to be in opposition to its limited oxidative capacity.

We propose the species *Acetobacter acidophilum* as a provisional name to be examined by taxonomists. Our feeling is that this organism owes its identity not so much to the presence or lack of a specialized set of enzymes, but to a very specialized interaction with the external medium.

What we have said about *Acetobacter acidophilum* does not exclude the fact that bacteria of very different types might be important in some industrial vinegar processes; our study concerns only bacteria which originated from one factory. The question of the bacteria involved in the 'quick vinegar process' was reviewed by Shimwell (1954). This author succeeded in the isolation by plating of an organism able to carry out good acidification. These bacteria were isolated on an ordinary medium and were able to grow in the presence of $CaCO_3$ on many different media. They possessed the characteristics of *A. mesoxydans*, as described by Frateur (1950), and are very different from *A. acidophilum.*

It is proposed to deposit this culture with the National Collection of Industrial Bacteria, Teddington, England.

This work was supported by the Union Belge des Vinaigriers and the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (I.R.S.I.A.). We owe to the Vinaigrerie l'Etoile and to its Director the late Mr R. Dothey, not only the material, but also the encouragement to examine this industrial problem.

A short report on this subject was given at the Congrès International du Vinaigre, La Haye, May 1957, by J. M. Wiame, R. G. Dothey and R. Harpigny.

REFERENCES

AJL, S. J. & WERKMAN, C. H. (1948). Replacement of CO_2 in heterotrophic metabolism. *Arch. Biochem.* **19**, 483.

Bergey's Manual of Determinative Bacteriology (1948). 6th edition. Ed. by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. Baltimore: The Williams and Wilkins Company.

Bergey's Manual of Determinative Bacteriology (1957). 7th edition. Ed. by Breed, R. S., Murray, E. G. D. & Smith, N. R. Baltimore: The Williams and Wilkins Company.

FRATEUR, J. (1950). Essai sur la systématique des acetobacters. *Cellule*, **53**, 287.

HENNEBERG, W. (1926). *Handbuch der Gärungsbakteriologie*. Berlin: Paul Parey.

HROMATKA, O. & EBNER, H. (1949). Untersuchungen über die Essiggärung. I. Fesselgärung und Durchlüftungsverfahren. *Enzymologia*, **13**, 369.

HROMATKA, O. & EBNER, H. (1950). Untersuchungen über die Essiggärung. II. Sauerstoffmangel der Fesselgärung. *Enzymologia*, **14**, 96.

KINGSBURY, J. M. & BARGHOORN, E. S. (1954). Silica gel as a microbiological medium: potentialities and a new method of preparation. *Appl. Microbiol.* **2**, 5.

LAMBION, R. & WIAME, J. M. (1951). Cultures pures d'acetobacter dans les conditions d'acétification rapide. *Rev. Ferment.* **6**, 25.

LWOFF, A., AUDUREAU, A. & MONOD, J. (1947). Essais d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. *Ann. Inst. Pasteur*, **73**, 323.

SHIMWELL, J. L. (1954). Pure culture vinegar production. *J. Inst. Brew.* **60**, 136.

WIAME, J. M. (1957). Le rôle biosynthétique du cycle des acides tricarboxyliques. *Advanc. Enzymol.* **18**, 241.

WIAME, J. M. & BOURGEOIS, S. (1955). Le rôle de l'anhydride carbonique dans les croissances bactériennes. *Biochim. biophys. Acta*, **18**, 269.

WIAME, J. M. & LAMBION, R. (1950). Contribution à l'étude des acetobacters de vinaigrerie du type 'rapide'. *Rev. Ferment.* **5**, 209.

WIAME, J. M. & STORCK, R. (1953). Métabolisme de l'acide glutamique chez *Bacillus subtilis*. *Biochim. biophys. Acta*, **10**, 268.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Colonies of *Acetobacter acidophilum* on Winogradsky silica gel medium.

Fig. 2. Microscope view of young colonies of *A. acidophilum* on solid media made of Ludox (×110).

Fig. 3. Microscope view of very young colonies of *A. acidophilum* on solid media made of Ludox (×220).

Fig. 4. Edge of a colony of *A. acidophilum* (×440).

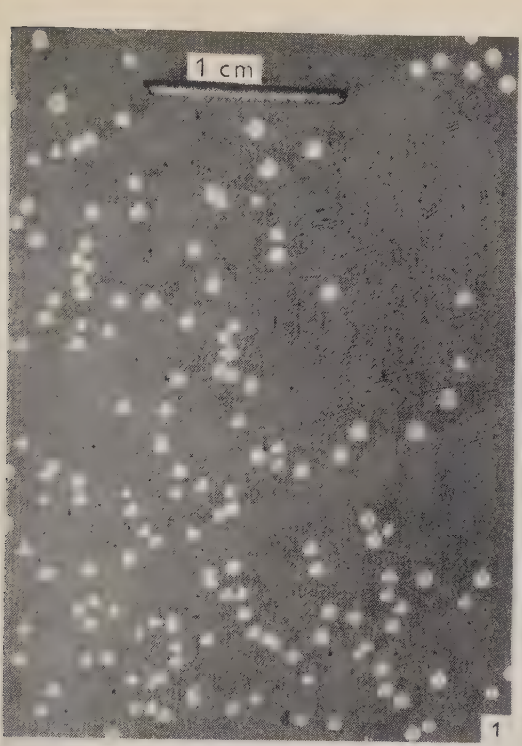
PLATE 2

Fig. 5. Phase contrast aspect of the bacteria (×770).

Fig. 6. Electron microscope photograph of *A. acidophilum* after growth in liquid medium, washing, fixation in formol.

Fig. 7. Electron microscope photograph of *A. acidophilum* (as Fig. 6).

(*Received* 26 *August* 1958)



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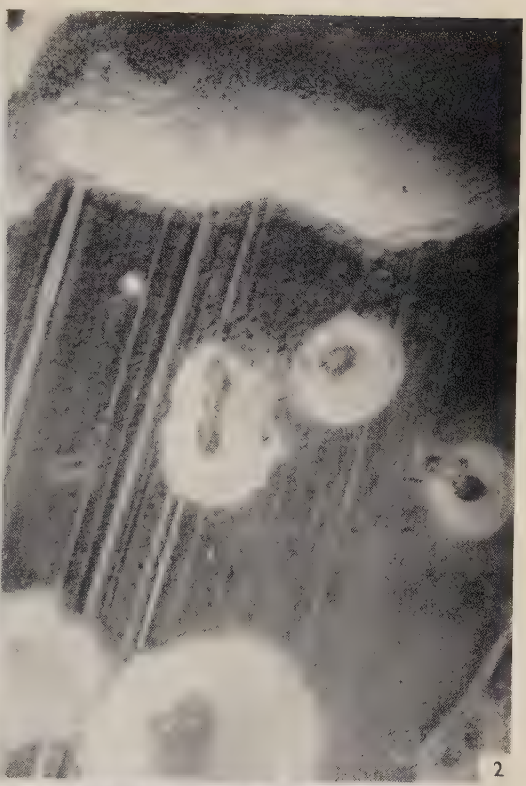

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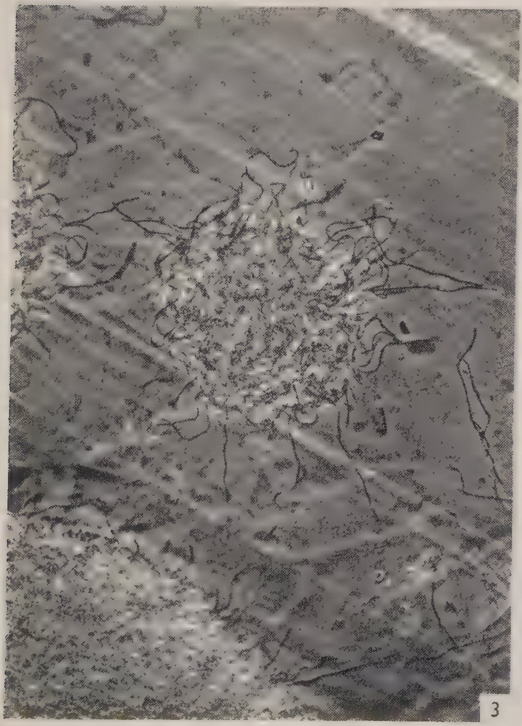

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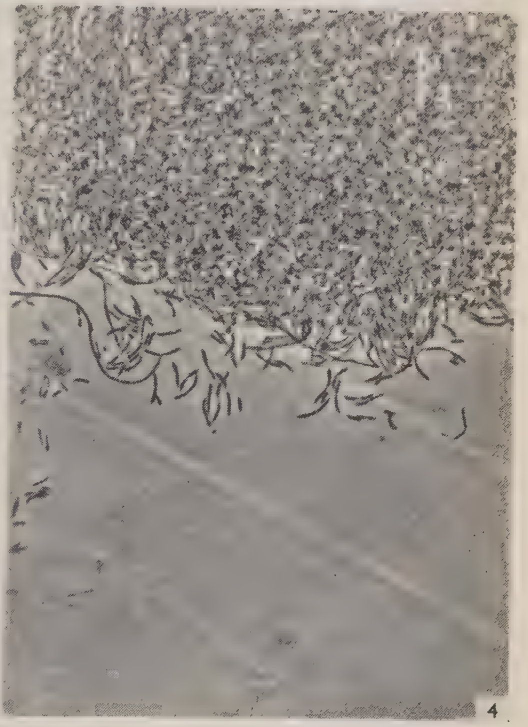

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J. M. Wiame, R. Harpigny & R. G. Dothey —A new type of Acetobacter.
Plate 1

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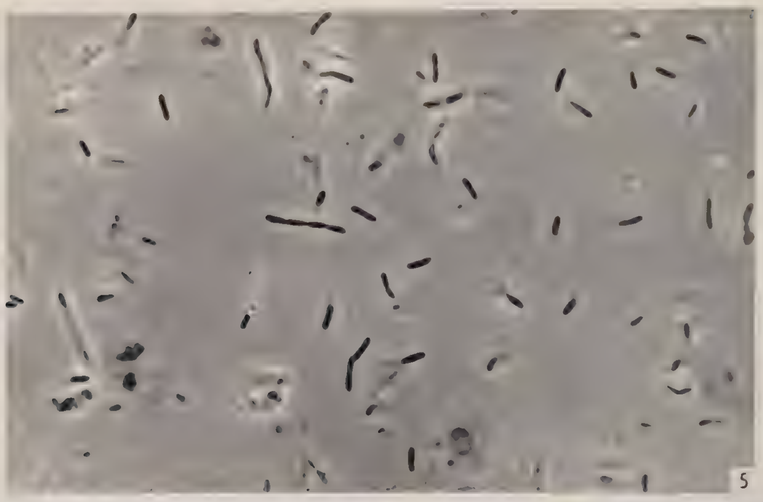

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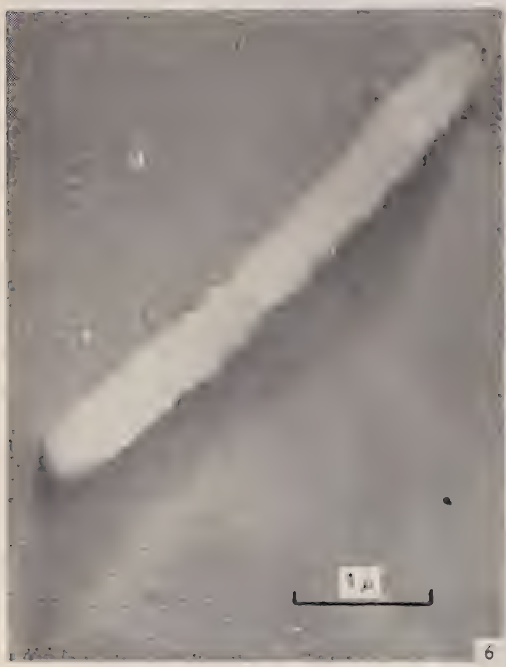

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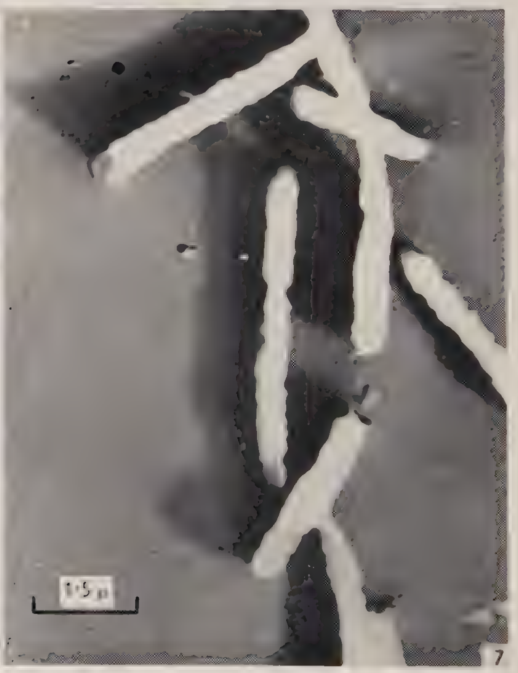

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J. M. Wiame, R. Harpigny & R. G. Dothey—A new type of Acetobacter.
Plate 2

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Aoyagi, T. & Mizuno, D. (1959). *J. gen. Microbiol.* 20, 173–179

Cultivation of Mycobacteria Rocked in Non-Protein Medium Containing a High Concentration of Tween 80

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National Institute of Health, Tokyo, Japan

SUMMARY: Mycobacteria were grown in rocked ⊥ tubes (Monod) in a medium which contained asparagine, glycerol, 1·0% (w/v) Tween 80 and no albumin fraction V. This permitted abundant well-dispersed diffuse growth. The amount of growth could be measured photometrically.

Smith (1947) reported turbidimetric observation of the growth of mycobacteria by using the medium devised by Dubos & Davis (1946) and Dubos & Middlebrook (1947) which contained Tween 80 and albumin fraction V in a modified Kirchner's medium and allowed a diffuse growth of the organism. Volk & Myrvik (1953), Halpern & Kirchheimer (1954) and Miller & Roessler (1956), reporting similar submerged cultures of mycobacteria either by aeration or by agitation, observed the logarithmic growth of the bacilli and obtained a good yield of organisms. However, all these media except that of Miller & Roessler (1956) contained albumin fraction V.

A ⊥-shaped culture tube devised by Monod (see Monod, Cohen-Bazire & Cohn, 1951; van Heyningen & Gladstone, 1953) was modified by us giving good results for rocked cultures of bacteria (Matsumura, Aoyagi & Mizuno, 1956); the mechanical damage of bacteria was very small at good rates of oxygen diffusion into the medium. The present paper deals with the diffuse growth of various strains of mycobacteria in a defined medium containing a high concentration of Tween 80 but without albumin fraction V, by cultivation in rocked ⊥ tubes. The requirements for nitrogen and carbon sources were examined for these strains. A preliminary account of some of the work has already been published in Japanese (Aoyagi & Mizuno, 1956).

METHODS

Organisms. Mycobacterium tuberculosis strains H37Rv, H2, Imamura (avirulent human type); BCG; *M. avium* A–62; *M. phlei*; *M. smegmatis* were used (all these strains were supplied by the Department of Tuberculosis of this Institute).

Media. The liquid medium, a modified Sauton medium, used in these experiments had the following composition (g.): asparagine, 4·0 or sodium glutamate, 4·0; citric acid, 2·0; K_2HPO_4, 0·5; $MgSO_4.7H_2O$, 0·5; ferric ammonium citrate, 0·05; glycerol, 60 ml. or glucose, 20. These materials were dissolved in 980 ml. distilled water, adjusted to pH 7·2 and autoclaved. When glucose was used it was dissolved separately in distilled water (20 g./50 ml.) steamed 3 times

Table 2. *The generation time of various mycobacteria in rocked culture using glycerol as a carbon source*

	Nitrogen source			
	Asparagine		Glutamate	
Strain	Pre-incubation medium	Generation time (hr.)	Pre-incubation medium	Generation time (hr.)
H37Rv	Asp+gly	28	Asp+gly	—
	Asp+gluc	20	Glut+gluc	28
	Glut+gluc	28		
H2	Asp+gly	25	Asp+gly	35
	Asp+gluc	27	Asp+gluc	30
Imamura	Asp+gly	22	Asp+gluc	22
	Asp+gluc	20		
BCG	Asp+gly	28	—	—
M. avium (A–62)	Asp+gly	5	Glut+gly	25
M. phlei	Asp+gly	4	Glut+gly	10
M. smegmatis	Asp+gly	3	Glut+gly	3

Asp = asparagine; glut = glutamate; gly = glycerol; gluc = glucose.

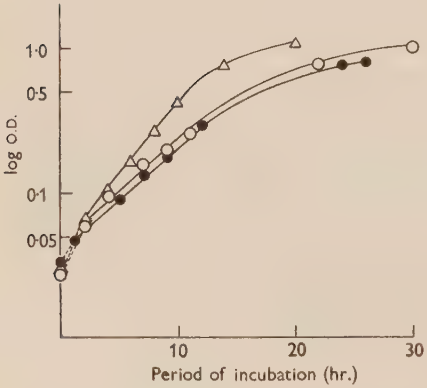

Fig. 3

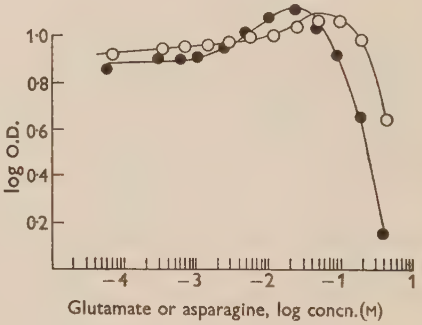

Fig. 4

Fig. 3. The growth curve of various strains of mycobacteria. ○—○, *Mycobacterium avium* (A–62); ●—●, *M. phlei*; △—△, *M. smegmatis*.

Fig. 4. The relationship between the concentration of glutamate and of asparagine and the growth of strain Imamura at 100 hr. The carbon source was glucose. ○—○, asparagine; ●—●, glutamate.

the BCG strain glutamate + glycerol and asparagine + glucose did not give good growth on the surface subcultures for inoculum and only glutamate + glucose and asparagine + glycerol showed good growth in the test-rocked cultures.

The effect of different concentrations of asparagine and of sodium glutamate in the presence of glucose is shown in Fig. 4 and of glycerol and glucose in the presence of asparagine in Fig. 5 for the Imamura strain. Similar relations were observed with the other strains. The relationship between the total viable units and optical density during growth is shown in a typical case (Fig. 6).

Subcultures. Every strain examined could be subcultured serially more than three times by using as inoculum 0·5 ml. of culture in the logarithmic phase. In the case of the Imamura strain (avirulent human type) and *Mycobacterium smegmatis* more than 25 serial subcultures were carried out in the asparagine + glucose medium. No loss of acid fastness with the Imamura strain was observed after 10 serial subcultures, but a slight decrease was observed after 20 subcultures; *M. smegmatis* showed marked decrease of acid fastness after 10 subcultures. No significant differences of generation time or of growth curve were observed all through the subcultures both for Imamura and *M. smegmatis*. Electron microscopy showed no differences from the original form when organisms of the Imamura strain were observed at the 10th and 25th serial subcultures.

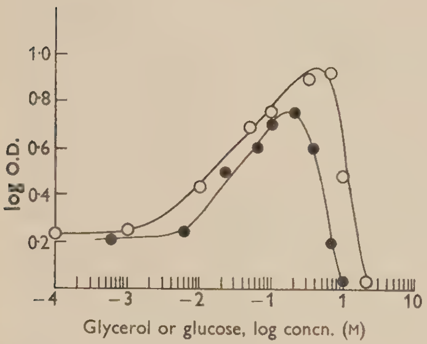

Fig. 5

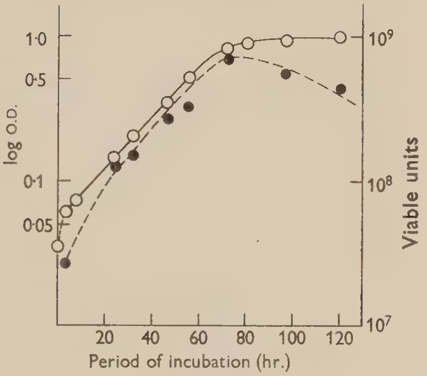

Fig. 6

Fig. 5. The relationship between the concentration of glucose and of glycerol and the growth of strain Imamura at 100 hr. The nitrogen source was asparagine. ○—○, glycerol; ●—●, glucose.

Fig. 6. The growth curve of viable units and of optical density. Imamura strain was used. ○—○, optical density; ●—●, viable units.

DISCUSSION

Media for submerged diffuse culture of mycobacteria have till now contained albumin fraction V to antagonize the toxic effect of free oleic acid possibly contained in Tween 80, especially when using very small inocula. Bloch & Noll (1953) observed the loss of acid fastness of strain H37Rv in cultures containing high concentrations of Tween 80. Minami (1957) showed a morphological change of *Mycobacterium avium* (Takeo strain) in the presence of oleic acid (10^{-3}M). In the present work Tween 80 at the concentration of 0·7–1·0% (w/v) (equiv. 10^{-4}M free oleic acid in our preparation) did not show the above toxicity nor morphological changes. This was probably partly due to the size of our inoculum (Davis, 1948), which could annul the toxic effect of oleic acid, and also to the annulling effect of high concentrations of

Tween 80 against oleic acid toxicity (Davis & Dubos, 1947; Camien & Dunn, 1957; Minami, 1957). The viable count during growth showed no significant drop, at least not during the exponential phase.

That we could measure the degree of bacterial growth with a conventional photometer by placing the ⊥ tube directly in it at any time enabled us to estimate generation time very rapidly; this has hitherto been difficult to estimate easily (e.g. Youmans, 1946; Youmans & Youmans, 1949; Volk & Myrvik, 1953; Halpern & Kirchheimer, 1954).

Kull & Grimm (1952) reported that the growth of *Mycobacterium tuberculosis* was inhibited by agitation or by aeration in submerged cultures. We had evidence that vigorous agitation and aeration can damage bacteria (*Staphylococcus aureus*) mechanically especially in the early phase of the growth (Matsumura *et al.* 1956). The present work with ⊥ tubes used a mild rocking motion and gave sufficient aeration for luxuriant growth. When we used the medium of Dubos in ⊥ tubes white precipitates, possibly denatured albumin, sometimes appeared and interfered with the observation of optical density. For this reason a non-protein medium is desirable for rocked cultures. Under the conditions used the best carbon and nitrogen sources as regards growth rate were asparagine + glucose and the best as regards final yield of bacteria were asparagine + glycerol. The relatively heavy inocula used with these media made it difficult to determine the lag period of the growth curve, because of the period during the early stages of incubation (several hours, depending on the strain) while the bacterial suspension of the inoculum was becoming uniformly dispersed. To the naked eye the bacterial suspension appeared uniform but staining showed groups of 2 or 3 to more than 20 bacteria, while about one third of the organisms were always found separated from each other. Tween 80, among other surface-active agents examined (including Triton WR-1339) had the best homogenizing effect.

The advice of Dr T. Hashimoto is gratefully acknowledged.

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REFERENCES

Aoyagi, T. & Mizuno, D. (1956). A shake culture of acid-fast bacteria. *Proc. Jap. Bact. Soc.* In *Jap. J. Bact.* **11**, 629.

Bloch, H. & Noll, H. (1953). Studies on the virulence of tubercle bacilli variation in virulence effect by Tween 80 and thiosemicarbazone. *J. exp. Med.* **97**, 1.

Camien, M. N. & Dunn, M. S. (1957). Saturated fatty acids as bacterial antimetabolites. *Arch. Biochem. Biophys.* **70**, 327.

Davis, B. D. (1948). Absorption of bacteriostatic quantities of fatty acids from media by large inocula of tubercle bacilli. *Publ. Hlth Rep. Wash.* **63**, 455.

Davis, B. D. & Dubos, R. J. (1947). The binding of fatty acids by serum albumin, a protective growth factor in bacteriological media. *J. exp. Med.* **86**, 215.

Dubos, R. J. & Davis, B. D. (1946). Factors affecting the growth of tubercle bacilli in liquid media. *J. exp. Med.* **83**, 409.

Dubos, R. J. & Middlebrook, G. (1947). Media for tubercle bacilli. *Amer. Rev. Tuberc.* **56**, 334.

Halpern, B. & Kirchheimer, W. F. (1954). Studies on the growth of *Mycobacteria*. II. The effect of oxygenation and aeration on the growth pattern of *Mycobacteria*. *Amer. Rev. Tuberc.* **70**, 665.

Heyningen, W. E. van & Gladstone, S. P. (1953). The neurotoxin of *Shigella shigae*. 3. The effect of iron on production of the toxin. *Brit. J. exp. Path.* **34**, 221.

Kull, F. C. & Grimm, M. R. (1952). Growth of *Mycobacterium tuberculosis* var. *hominis* in agitated culture. *J. Bact.* **64**, 431.

Matsumura, G. Aoyagi, T. & Mizuno, D. (1956). Shaken culture by Monod type. *Kagaku-no-Ryoiki* (*J. Jap. Chem.*), **10**, 65.

Miller, I. L. & Roessler, W. G. (1956). Growth of *Mycobacterium tuberculosis* in liquid media. *Amer. Rev. Tuberc.* **73**, 716.

Minami, K. (1957). Bactericidal action of oleic acid for tubercle bacilli. II. Morphological response. *J. Bact.* **73**, 345.

Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*, la specificité de l'induction. *Biochem. Biophys. Acta*, **7**, 585.

Smith, D. G. (1947). Quantitative measurement of growth of *Mycobacterium tuberculosis*. Effect of streptomycin. *Proc. Soc. exp. Biol., N.Y.* **64**, 36.

Volk, W. A. & Myrvik, Q. N. (1953). An explanation for the arithmetic linear growth of mycobacteria. *J. Bact.* **66**, 386.

Youmans, G. P. (1946). A method for the determination of culture cycle and the growth rate of virulent human type tubercle bacilli. *J. Bact.* **51**, 703.

Youmans, G. P. & Youmans, A. S. (1949). A method for the determination of the rate of growth of tubercle bacilli by the use of small inocula. *J. Bact.* **58**, 247.

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Aoyagi, T. & Mizuno, D. (1959). *J. gen. Microbiol.* **20**, 180–183

A Rapid Antibacterial Test for Mycobacteria Using a Reverse T Type of Tube in Rocked Culture

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SUMMARY: Antibacterial tests with mycobacteria were carried out in rocked ⊥ tubes (Monod). The medium used was Sauton medium + 1 % (w/v) Tween 80. It took 48 hr. to get a reproducible value for the ID50 dose (50 % inhibition dose) with tubercle bacilli of human type, and 8 hr. with the avian type.

Antibacterial tests with mycobacteria may take a long time, for instance several weeks in the case of human tubercle bacilli. When an acid-fast saprophyte is used for shortening the time for the test, the results obtained sometimes cannot be translated directly as measures of the effect of the drug on human type organisms. We reported (Aoyagi & Mizuno, 1959) that various strains of mycobacteria could be grown in a non-protein medium in rocked ⊥ tubes and that mycobacteria could be treated conventionally for the determination of growth curves by observations of the optical density (OD) of cultures, with the use of suitable calibration curves to give absolute measures of amounts of growth. The present paper deals with an antibacterial test for mycobacteria with this type of rocked culture, estimating the ID50 dose (50 % inhibition dose) by the method of Treffers (1956) where two or three dilutions of drug are sufficient, and observing the growth of bacteria in the logarithmic phase. The ID50 dose can be read from graphs obtained by taking drug concentration plotted on a logarithmic scale as abscissa and probit of % inhibition as ordinate.

METHODS

Organisms. Mycobacterium tuberculosis var. *hominis* strain H37Rv and *M. avium* strain A–62 were used; details are given in the preceding paper (Aoyagi & Mizuno, 1959).

Medium. This was prepared as follows: asparagine, 4·0 g.; citric acid, 2·0 g.; K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; ferric ammonium citrate, 0·05 g.; dissolved in 930 ml. distilled water, adjusted to pH 7·2 and autoclaved. Separately, 40 % (w/w) glucose solution was prepared and sterilized at 100° for 30 min. on three successive days. To each 9·3 ml. of basal medium was added 0·5 ml. glucose solution. Tween 80 solution (0·2 ml. 50 %, w/v; autoclaved) was then added to give a final Tween 80 concentration of 1·0 %. Other details were given in the preceding paper.

Incubation and inoculum. The same procedure as described previously (Aoyagi & Mizuno, 1959) was used.

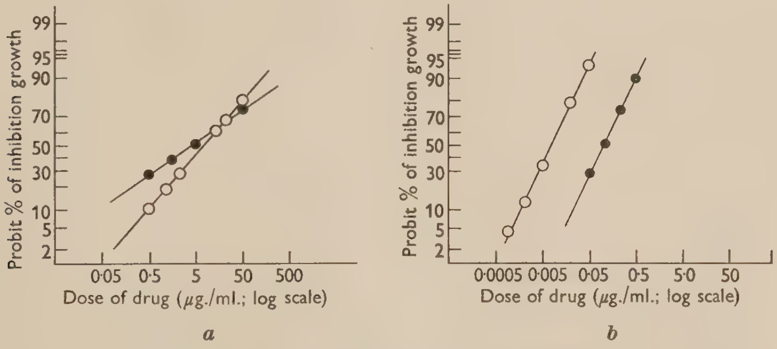
Drugs test. Crystalline dihydrostreptomycin sulphate (Meiji); viomycin (Parke, Davis); sodium *p*-aminosalicylate (Tanabe); Tibion (Takeda); pyrazinamide (Sankyo); levulinic acid oxime (Institute of Applied Microbiology, University of Tokyo); *iso*nicotinic acid hydrazide (Tanabe); angstomycin (Institute of Applied Microbiology, University of Tokyo); *N*-hydroxy-2-pyridine thione (Tanabe); pyridomycin (Sankyo).

Determination of ID 50 dose. According to Treffers (1956) the ID 50 dose of a drug may be determined by intercepting a 50 % inhibition, taking as abscissa drug concentration plotted on a logarithmic scale and as ordinate probit of % inhibition. The % inhibition was determined by comparing the growth of the organisms with and without drug during the logarithmic phase of growth by observations of the optical density of the cultures in the rocked ⊥ tubes. Inhibitions of more than 90 % and less than 10 % were not used for the ID 50 dose determination.

RESULTS

ID 50 of several drugs against tubercle bacilli

Figure 1*a* shows the normal response curve for *Mycobacterium avium* strain A–62 with dihydrostreptomycin and viomycin. The time needed to determine the degree of growth inhibition was 8 hr. The same procedure was followed for *M. tuberculosis* strain H 37 Rv (Fig. 1*b*), the time needed to obtain the response curve being 48 hr. Other antituberculous drugs were tested by this procedure to estimate the ID 50 dose against *M. avium* A–62 and *M. tuberculosis* H 37 Rv; the results are shown in Table 1.



a *b*

Fig. 1*a*. The relationship between the drug concentration (plotted on logarithmic scale) and the probit of % inhibition of bacterial growth. ○—○, dihydrostreptomycin; and ●—●, viomycin. The ID 50 dose can be obtained from this linear relation at the ordinate intercept of a 50% end point. The slopes for viomycin and dihydrostreptomycin are 0·60 and 1·30, respectively. The organism used was *Mycobacterium avium* A–62.

Fig. 1*b*. The same as Fig. 1*a*, except that the organism used was *M. tuberculosis* strain H37 Rv. The slope is 2·10 for both cases.

DISCUSSION

In the present work the antibacterial activity of drugs was determined during the logarithmic phase of growth; the amount of growth of the mycobacteria was observed directly by placing the ⊥ culture tubes (⊢) in the photometer.

Table 1. *The ID 50 doses of various drugs calculated by using the method of Treffers* (1956) *from the data obtained by measuring the growth of* Mycobacterium tuberculosis *H 37 Rv and* M. avium A–62 *in rocked ⊥ tube cultures*

	Test organism			
	Strain A–62		Strain H 37 Rv	
	ID 50 dose			
Drug	(*μ*g./ml.)	(M)	(*μ*g./ml.)	(M)
Dihydrostreptomycin	10	2×10^{-5}	0·01	2×10^{-8}
Viomycin	5	—	0·1	—
Na-*p*-aminosalicylate	> 500	$> 10^{-3}$	0·2	10^{-6}
Tibion	500	2×10^{-3}	1·0	5×10^{-6}
Pyrazinamide	2	2×10^{-5}	500	5×10^{-3}
Levulinic acid oxime	> 500	3×10^{-3}	100	5×10^{-4}
*Iso*nicotinic hydrazide	5	5×10^{-5}	0·05	4×10^{-7}
Angstomycin	> 500	—	> 500	—
N-Hydroxy-2-pyridine thione	100	10^{-3}	0·02	2×10^{-7}
Pyridomycin	> 500	—	> 500	—

The time needed to determine an ID 50 dose was short, both as regards the time interval between inoculation and observation of growth and the time needed to determine the amount of growth or the % inhibition. It took only 8 hr. with *Mycobacterium avium* and 48 hr. with *M. tuberculosis* strain H 37 Rv for reproducible data for antibacterial activity to be obtained.

The slope of the curve, the reproducibility, the determination of drug resistance and other problems were discussed by Treffers (1956) who said 'the drugs acting on the same species of organism, or one drug acting on two species, may, by coincidence, exhibit response curves of identical slope without warranting the conclusion that the mechanisms of action are identical, or even related in any way. A change in slope, with change in the distributions of susceptibilities is at least highly suggestive that some alteration in the mechanism of action has occurred.' The slopes for viomycin and dihydrostreptomycin in Fig. 1*b* (strain H 37 Rv) are the same (2·10). The value for streptomycin with *Escherichia coli* was 2·10 (Treffers, 1956). On the other hand, *Mycobacterium avium* strain A–62 showed different values, the slope for viomycin being 0·60 and for dihydrostreptomycin 1·30. At least it can be considered that the site of action of viomycin is different from that of dihydrostreptomycin with *M. avium* A–62.

The present results confirm that the strains used in testing must be carefully selected. Dihydrostreptomycin, sodium *p*-aminosalicylate, Tibion and *N*-hydroxy-2-pyridine thione showed strong activity against *Mycobacterium tuberculosis* strain H 37 Rv, while they did not show any marked activity against *M. avium*.

There are two defects in the present techniques. The optical density is used to determine the growth of organisms, so that it is rather tedious to test a coloured sample in a high concentration. Coloured control should be deducted in each determination. Since Tween 80 is used in a high concentration (1 %), an unspecific reversal (antagonism) and acceleration (synergism) cannot be denied (Mima, 1953; Youmans & Youmans, 1948). But so far as the drugs used in this experiment are concerned, no such reversal or acceleration was observed. The antagonizing effect of Tween 80 against antibacterial activity of drugs was very similar to that of serum albumin (Natori, 1958).

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REFERENCES

AOYAGI, T. & MIZUNO, D. (1959). Cultivation of mycobacteria rocked in non-protein medium containing a high concentration of Tween 80. *J. gen. Microbiol.* **20**, 173.

MIMA, H. (1953). Application of surface active agents. *J. Japan. Chem.* **7**, 689.

NATORI, S. (1958). Antibacterial effect of lichen substances and related compounds. VIII. Some observations on inactivation of antitubercular activity by surface active agents and serum. *Pharm. Bull.* **6**, 94.

TREFFERS, H. P. (1956). The linear representation of dosage-response curves in microbial-antibiotic assays. *J. Bact.* **72**, 108.

YOUMANS, A. S. & YOUMANS, G. P. (1948). The effect of Tween 80 *in vitro* on the bacteriostatic activity of twenty compounds for *Mycobacterium tuberculosis*. *J. Bact.* **56**, 245.

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Moses, V. (1958). *J. gen. Microbiol.* **20**, 184–196

[^{14}C]Glucose Metabolism in Fungal Cells

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SUMMARY: The incorporation pattern of ^{14}C from [^{14}C]glucose by the fungus *Zygorhynchus moelleri* has been investigated. The following substances became labelled in incubation periods of 6 sec. to 30 min.: the monophosphates of glucose, fructose, sedoheptulose, ribose, maltose, glycerol, gluconic acid, glyceric acid, enolpyruvic acid and guanosine; the diphosphates of fructose, glucose, glyceric acid, adenosine, uridine, inosine and guanosine; the triphosphates of adenosine and uridine; uridinediphosphoglucose and uridinediphosphoribose; free maltose and fructose; aspartic acid, glutamic acid, alanine, valine, tyrosine, proline, histidine, threonine, citrulline and glutamine; malic acid, citric acid, succinic acid, fumaric acid and glyceric acid. A small percentage of the ^{14}C was present in unidentified substances. The kinetics of the incorporation of ^{14}C, first into uridinediphosphoglucose, then into maltose phosphate, and finally into free maltose, suggests this sequence of compounds for the biosynthetic pathway of maltose formation.

The main effects of starvation of the cells by shaking them in phosphate buffer for 24 hr. were an increased % incorporation of ^{14}C into maltose and the sugar phosphates, and a decreased % incorporation into amino acids, organic acids and nucleotides. The addition of a small amount of ammonia to starved cells increased the absolute rate of incorporation of ^{14}C from labelled glucose into soluble cellular constituents; however, while the % of the total soluble ^{14}C found in the nucleotides was markedly increased when ammonia was added, the % in the sugar phosphates decreased. In the presence of azide, the incorporation of ^{14}C by starved cells into maltose and the nucleotides was severely inhibited and the incorporation into amino acids was somewhat increased. Thus, one of the most important results of starvation was to prevent incorporation of ^{14}C into the nucleotides, organic acids and amino acids, while the addition of ammonia overcame the effects of starvation and promoted nucleotide synthesis. Ammonia had very little effect on the cells in the presence of azide.

A kinetic study of the sequence in which ^{14}C was detected in these compounds indicated that glucose was metabolized by both the glycolysis and the oxidative pathways, and later by the tricarboxylic acid cycle.

Investigation into the respiratory activities of fungi with labelled substrates has been used mainly to study the distribution of label within various molecules produced enzymically from substrates labelled in known positions. From the results it has been possible to arrive at certain conclusions about the importance of particular pathways for the metabolism of the relevant substrates. Thus, Goldschmidt, Yall & Koffler (1956) and Moses (1957) demonstrated that the tricarboxylic acid cycle plays a prominent role in the oxidation of acetate in two moulds, and Heath & Koffler (1956) and Bartlett & Moses (1957) used variously labelled glucoses to investigate the importance of the oxidative and fermentative pathways in the utilization of glucose by *Penicillium chrysogenum* and *Zygorhynchus moelleri*, respectively.

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Relatively little work has been done, however, along the lines of Calvin (1956) and co-workers, in which an organism is fed a labelled substrate for short periods of time and then killed and extracted to determine the nature of all the substances incorporating the label from the original substrate. Claridge & Werkman (1954) studied the incorporation of label from $[^{14}C]$2-ketogluconate by *Pseudomonas aeruginosa*, and Milhaud & Aubert (1955) supplied labelled glucose and ethanol to baker's yeast. In both cases a considerable number of products were detected on a subsequent analysis of the cells, and an attempt was made to obtain a picture of the kinetic relations between the compounds observed. A preliminary study by Bartlett & Moses (1957), using *Zygorhynchus moelleri*, showed that this fungus converts $[^{14}C]$ glucose to a large number of compounds in a very short time (15 sec. was the shortest incubation time used), but they did not identify many of the products of glucose metabolism, and no very definite conclusions were drawn.

The present communication reports a further attempt to elucidate this problem by using cells of *Zygorhynchus moelleri* in various experimental conditions, and analysing the cells after several short periods of exposure to very high specific activity $[^{14}C]$glucose. Following the killing and extraction of the cells with hot ethanol, the extracts were analysed by chromatography and radioautography as described by Benson, Bassham, Calvin, Goodale, Haas & Stepka (1950). The results are consistent with the operation of both the hexose monophosphate shunt and the glycolysis mechanisms for glucose breakdown, and with the subsequent involvement of the citric acid cycle. Considerable differences were found in the pattern of incorporation of ^{14}C as between starved and fresh cells, and a study has been made of the effects of sodium azide and of ammonium chloride on glucose metabolism in the starved cells.

METHODS

Cultures of *Zygorhynchus moelleri* (Commonwealth Mycological Institute No. 21994) were grown in a medium similar to that used in previous studies (Moses, 1954), but with yeast or liver extracts omitted. Spores obtained from several agar slope cultures of the fungus were inoculated into 200 ml. of the growth medium in a 2 l. bottle which was shaken vigorously on a reciprocal shaker for 16–18 hr at room temperature (*c.* 25°). After growth the organism was harvested by centrifugation, washed with distilled water, and subsequently resuspended in distilled water at a concentration of about 4 ml. wet packed organisms/100 ml. suspension. The volume of the wet packed organisms was determined by centrifuging the suspension in a graduated 12 ml. conical tube for 5 min. at 500 ***g***.

When starved cells were required, the hyphae, after harvesting and washing, were resuspended in 200 ml. of 0·067 M-phosphate buffer (pH 6·8) and were shaken for a further 24 hr. They were then washed and resuspended in distilled water.

Each of several 50 ml. Erlenmeyer flasks received 5 ml. cell suspension. At the beginning of the incubation period 40 μl. of $[^{14}C]$glucose solution (specific activity 240 μc./mg.; 33·6 μc./flask; final glucose conc. $1{\cdot}6 \times 10^{-4}$M) was rapidly injected into one flask at a time. After the desired incubation period at room temperature the suspension was quickly poured on to a sintered glass funnel bearing a layer of Celite filter aid and inserted through a rubber stopper into the top of a Buchner flask. The liquid of the suspension was sucked through the filter by a water pump, the funnel and the rubber stopper removed from the Buchner flask, inserted into a centrifuge tube of suitable size, and 5 ml. of boiling 80 % (v/v) ethanol in water poured on to the organism on the sintered glass disk. The operations of filtering and killing the cells were performed as quickly as possible, and, with practice, only about 2 sec. elapsed between pouring the suspension on to the filter-pad and the addition of the hot aqueous ethanol.

The cells were allowed to stand for 2 hr. at room temperature, the funnel loosened in the centrifuge tube, and the liquid of the suspension forced through the filter-disk and Celite pad into the tube by pressure from a N_2 cylinder. The funnel was again inserted into the tube and 2 ml. of boiling 20 % (v/v) ethanol in water poured on to the organism. After 1 hr. the second extract was filtered and the process repeated once more with a further 2 ml. of boiling 20 % (v/v) ethanol in water.

The three ethanol extracts from each sample were pooled and evaporated *in vacuo* below 40° to a small volume. Two-dimensional descending chromatograms were run of each extract on 18 in. × 22 in. sheets of Whatman No. 4 filter-paper previously washed with 2 % (w/v) oxalic acid. Phenol + water was used as the solvent in the first dimension and *n*-butanol + propionic acid + water as that in the second dimension (Benson *et al.* 1950). Generally, two chromatograms were run for each sample. For the first, one-fifth of each extract was chromatographed so that each solvent just reached the edge of the paper (8–10 hr. for phenol + water, and 7–8 hr. for *n*-butanol + propionic acid + water) to obtain a picture of all the soluble substances in the extract. On such chromatograms sugar phosphates and nucleotides, important compounds in this type of investigation, move but a small distance from the origin, and the separation of many of these compounds is very poor. To overcome this difficulty, a further two-fifths of each extract was chromatographed for 24 hr. with phenol + water and for 20 hr. with *n*-butanol + propionic acid + water (Moses & Calvin, 1958). This spread out the area which contained the sugar phosphates and nucleotides much further, and good separation was usually achieved. The final two-fifths of each extract was kept in reserve in case one of the chromatograms was unsatisfactory. The locations of the radio-active materials were determined by exposing the chromatograms to 14 in. × 17 in. sheets of single-coated blue-sensitive Du Pont X-ray film 507-E for a suitable period. Radio-active ink markers were stamped on the corners of the chromatograms to facilitate the subsequent alignment of the film with the paper.

The ^{14}C present in each compound on the chromatogram was determined with a Scott-type large window Geiger–Müller tube connected to a scaler. The window was of Du Pont 'Mylar' Polyester film and the counting tube was flushed continuously with 'Q gas' (99·05 % (v/v) He and 0·95 % (v/v) *iso*butane).

When this counter was used to count ^{14}C from Whatman No. 4 filter-paper, about 6–7 % of the total disintegrations were counted. The spots were usually counted on both sides of the paper and the values obtained were averaged.

The identity of labelled compounds on the chromatograms was established partly by their position and their susceptibility to hydrolysis by acid phosphatase, and was confirmed by co-chromatography with authentic marker substances, when necessary after removal of the phosphate groups from the unknown substances. Co-chromatography was performed in the same solvent systems as those mentioned above. Exact coincidence between the labelled substance and the authentic marker was taken as the criterion of identity. The following techniques were used for the location of the authentic markers. Amino acids were sprayed with 0·1 % (w/v) ninhydrin in 95 % (v/v) ethanol in water and the chromatograms heated at 100° for 5 min. Organic acids were sprayed with a 0·04 % (w/v) ethanolic solution of bromcresol green, made just alkaline with a little ammonia. Sugars and other reducing substances (e.g. glyceric acid) were detected by dipping the chromatograms into an acetone solution of $AgNO_3$, allowing them to dry in air, and then spraying with ethanolic NaOH. After development of the spots, the excess $AgNO_3$ was washed off in ammonia solution and the papers dried (Trevelyan, Procter & Harrison, 1950). Ketoses were also detected with the orcinol + trichloroacetic acid reagent (modified from Klevstrand & Nordal, 1950). Nucleosides were located by their quenching of fluorescence under u.v. irradiation.

Spots suspected of being phosphates were eluted from the paper with water, treated either with purified Polidase S (Cohen, 1953) + acetate buffer (pH 5), or with human seminal acid phosphatase (a gift from Professor H. A. Barker) + acetate buffer (pH 5) and allowed to stand overnight at 37°. Authentic markers were added, and the substances chromatographed.

Labelled glucose was prepared from *Canna* leaves allowed to carry out photosynthesis in the presence of $^{14}CO_2$ (Putman & Hassid, 1952). Owing to the radiation decomposition of the very high specific activity glucose, samples of the glucose were chromatographed immediately before use, and then eluted from the chromatograms. This achieved a satisfactory purification of the substrate and, except for one experiment in which this was not done (see Results) no interference from contaminants in the [^{14}C]glucose was apparent on the chromatograms.

RESULTS

Identification of substances formed from glucose

A considerable number of substances were labelled from [^{14}C]glucose by the hyphae, some in very short periods. Aspartic acid, glutamic acid, alanine, valine, tyrosine, glutamine, proline, histidine, threonine, citrulline, malic acid, citric acid, succinic acid, fumaric acid, glyceric acid, maltose and fructose were identified by elution and co-chromatography with appropriate authentic markers. The maltose spot contained only glucose after acid hydrolysis (N-HCl at 60° for 12 hr.).

Analysis of the area on the chromatograms known to contain the sugar monophosphates (Benson *et al.* 1950; Bassham & Calvin, 1957) was performed by treatment with phosphatase followed by co-chromatography. Phosphoglyceric acid, phosphoenolpyruvic acid, ribose monophosphate, glycerol phosphate, maltose phosphate and phosphogluconic acid were identified, after hydrolysis, by co-chromatography with glyceric acid, pyruvic acid, ribose, glycerol, maltose and gluconic acid, respectively. The area suspected of being mixed hexose and heptose monophosphates was shown to contain glucose, fructose and sedoheptulose monophosphates. Sedoheptulose lies chromatographically between glucose and fructose, and is not easy to detect in the presence of large amounts of the hexoses. To confirm its presence, the monophosphate area after treatment with phosphatase was heated with 0·1 N-HCl at 100° for 5 min. Sedoheptulose was partly converted into sedoheptulosan, which chromatographs quite separately from the hexoses, and was detected by the orcinol + trichloroacetic acid spray (Klevstrand & Nordal, 1950). The area containing the sugar diphosphates (Benson *et al.* 1950; Bassham & Calvin, 1957) was shown, after treatment with phosphatase, to contain overwhelmingly fructose, with traces of glucose, gluconic acid, glyceric acid and possibly sedoheptulose.

The spot labelled 'UDPG' in Pl. 1, figs. 1–2 was shown, after treatment with phosphatase, to contain uridine and glucose, and was probably uridinediphosphoglucose; the spot designated 'UDPR' similarly contained uridine and ribose, and may, by analogy with uridinediphosphoglucose, be uridinediphosphoribose. In neither case, nor with any of the other nucleoside derivatives identified, was any attempt made to determine whether the ^{14}C was present in the sugar, or the base of the nucleoside, or in both.

A number of spots were observed which, from their chromatographic positions and u.v. absorption, were suspected of being nucleotides. Those labelled 'ATP' and 'ADP' gave adenosine, uridine and ribose on treatment with phosphatase, and were possibly ATP and ADP with a uridine-sugar contaminant. The 'nucleotide diphosphate' spot contained uridine, inosine and probably guanosine, and chromatographically corresponded to the diphosphates of these compounds. Uridine triphosphate (UTP) contained only uridine and corresponded with the position of the triphosphate ester; another spot was identified in a similar way as being guanosine monophosphate. A further spot (called 'unknown nucleotide monophosphate') may have been xanthosine monophosphate but its identity was not confirmed. In addition to all these compounds which have been identified with a greater or lesser degree of certainty, the radio-autograms showed the presence of up to ten weakly active substances which were not identified: in most cases not more than 2–5 % of the total activity incorporated from [^{14}C]glucose appeared in these unidentified compounds.

Kinetic studies with unstarved cells

Freshly harvested organisms, suspended in distilled water, was incubated with [^{14}C]glucose for fifteen different incubation periods between 6 sec. and 3 min., and in another experiment for 30 min. The distributions of the activities in a

Samples (5 ml.) of unstarved cell suspension (containing 0·2 ml. wet packed cells) were incubated with [^{14}C]glucose (33·6 μc. and 0·8 μmoles/sample) for the periods stated. The cells were killed and extracted with ethanol, and the extracts chromatographed. The ^{14}C in each compound on the chromatogram was counted. The values given are expressed as the % of the total ^{14}C incorporated into all soluble substances from labelled glucose, which is present in each individual substance. The ^{14}C remaining in the residual glucose was excluded from all calculations.

	Incubation period (sec.)															
	6	8	10·5	17·5	31	45	61	75	90	106	120	135·5	151	167	180	1800*
Sugar diphosphates†	2·1	4·9	4·9	4·2	3·4	3·9	3·6	2·7	2·5	2·9	2·4	2·1	2·4	3·2	2·3	0·18
Sugar monophosphates‡	52·2	35·8	34·8	31·8	38·2	33·8	29·5	26·2	25·4	24·7	22·5	19·3	17·7	17·2	16·5	1·4
Phosphoglycerate	5·0	7·7	9·6	8·3	10·8	8·5	8·4	8·0	6·8	5·5	5·5	5·7	4·9	4·6	4·6	0·48
Phosphoenolpyruvate	6·1	6·6	3·9	2·7	2·7	2·4	2·1	1·7	1·5	1·3	1·5	1·7	1·1	1·3	1·1	0·10
Phosphogluconate	4·6	4·7	3·5	4·1	3·2	2·0	1·9	1·5	1·1	1·1	1·1	1·1	0·88	0·87	0·85	0·09
Ribose monophosphate	5·6	4·0	4·1	1·9	2·6	1·5	1·9	1·2	0·74	1·6	1·0	1·2	0·89	0·96	1·1	0·55
Glycerol phosphate	0·0	3·3	2·4	2·4	2·2	2·1	2·0	1·5	1·1	1·1	1·4	1·2	0·86	1·3	0·73	0·55
Maltose phosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·26	0·43	0·62	1·2	1·6	1·8	1·9	1·9	1·8	0·0
Uridinediphosphoglucose	0·89	1·6	1·1	2·2	2·5	3·1	3·3	3·5	6·0	3·9	5·2	4·6	4·4	4·2	4·4	1·1
Uridinediphosphoribose	0·0	0·0	0·0	0·41	0·62	0·66	0·94	1·2	1·1	1·1	1·5	1·3	1·4	1·0	1·1	0·44
Nucleotide diphosphates§	0·0	0·85	1·1	3·8	4·6	7·0	7·8	9·3	9·9	9·2	10·3	9·0	8·2	9·5	9·2	26·4
Guanosine monophosphate	0·0	3·1	2·7	1·9	2·6	3·6	2·4	2·4	1·9	1·8	1·7	2·1	1·6	1·5	1·0	0·67
Uridine triphosphate	0·0	0·0	0·0	0·0	0·10	0·44	0·33	0·37	0·52	0·64	0·87	0·92	0·86	0·96	1·2	1·1
Adenosine diphosphate	0·0	0·0	0·0	0·0	0·0	0·32	0·40	1·1	0·56	0·45	0·43	0·31	0·25	0·32	0·32	0·0
Adenosine triphosphate	0·0	0·0	0·0	0·0	0·0	0·32	1·1	0·42	1·7	3·5	1·4	1·0	1·0	0·93	1·1	0·0
Maltose	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	1·3	1·7	1·9	2·4	2·7	2·5	2·5	1·2
Succinate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·07
Fumarate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·02	0·0	0·03	0·16	0·11	0·14	0·16	0·84
Citrate	0·0	0·0	2·0	1·3	1·1	1·3	1·2	1·2	1·0	1·0	1·1	1·4	1·4	1·4	1·4	6·7
Malate	3·5	3·4	3·9	5·1	1·8	1·8	2·0	1·7	1·1	0·88	1·0	1·4	1·2	1·1	1·3	6·0
Aspartate‖	20·1	17·5	14·3	14·4	11·5	8·5	9·6	10·0	9·4	10·2	10·0	10·3	10·7	10·8	11·0	20·3
Glutamate	0·0	0·0	4·5	3·5	2·1	2·9	3·9	4·5	5·1	5·1	6·0	6·8	8·0	8·1	8·2	22·1
Alanine	0·0	7·5	7·3	12·0	10·1	14·5	16·3	18·6	17·5	16·9	17·6	19·0	20·7	20·2	20·9	4·1
Glutamine	0·0	0·0	0·0	0·0	0·0	0·0	0·0	1·6	1·4	2·1	2·1	2·0	3·1	2·3	2·5	1·6
Valine	0·0	0·0	0·0	0·0	0·0	0·38	1·0	0·55	0·63	0·82	0·83	1·1	1·7	1·6	1·8	0·62
Proline	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·35
Histidine	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·37
Tyrosine	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·40	0·25	0·41	0·36	0·41	0·48	0·49	0·42	0·30
Unknown substances	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·83	0·99	0·86	1·6	1·7	1·4	2·5	2·4
Total in sugar phosphates	75·6	67·0	63·2	55·4	63·1	54·2	49·7	43·2	42·8	39·4	37·0	34·1	30·6	31·3	29·0	3·3
Total in uridine sugars	0·89	1·6	1·1	2·6	3·1	3·8	4·2	4·7	7·1	5·0	6·7	5·9	5·8	5·2	5·5	1·5
Total in nucleotides	0·0	4·0	3·8	5·7	7·3	11·7	12·0	13·6	14·6	15·6	14·7	13·3	11·9	13·2	12·8	28·2
Total in free sugars	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	1·3	1·7	1·9	2·4	2·7	2·5	2·5	1·2
Total in organic acids	3·5	3·4	5·9	6·4	2·9	3·1	3·2	2·9	2·1	1·9	2·1	2·9	2·7	2·6	2·9	13·6
Total in amino acids	20·1	25·0	26·1	29·9	23·7	26·3	30·8	35·7	34·3	35·5	36·9	39·6	44·7	43·5	44·8	49·7
Total activity/100 μl. cells (dis./min. $\times 10^{-5}$)	0·58	0·63	1·01	1·87	2·87	4·36	4·98	5·59	6·20	8·36	8·46	9·91	14·7	16·1	16·9	28·5

* Separate experiment. † Contains diphosphates of fructose, glucose and glyceric acid. ‡ Contains monophosphates of glucose, fructose and sedoheptulose. § Contains diphosphates of uridine, inosine, and guanosine. ‖ Contains a contaminant from the radio-active glucose used as substrate.

number of different compounds are presented in Table 1. These values are expressed for each compound as % total activity present in compounds other than glucose in the soluble fraction of the cells, omitting radio-activity present in the residual substrate glucose; in addition, the total activities in terms of disintegrations/min. for a standard sample of the total soluble fraction are given.

The time sequence of the incorporation of ^{14}C from [^{14}C]glucose into the various substances detected is consistent with the operation of both the glycolytic and oxidative pathways. Thus, in the first few seconds ^{14}C appeared in the sugar monophosphates and diphosphates, in phosphogluconate, ribose monophosphate, phosphoglycerate, phosphoenolpyruvate, and in malate (Table 1). A small amount of UDPG was present. Aspartic acid was apparently very radio-active, but this was probably an artefact due to chromatographic coincidence of aspartic acid with a radio-active contaminant in the [^{14}C]glucose, as in this particular experiment the glucose had not first been purified chromatographically (see Methods). In other experiments it was found that the first three radio-active substances to appear were the sugar monophosphates, diphosphates and phosphoglyceric acid. With the exception of aspartic acid, four substances showed a fall in the percentage of the total ^{14}C from the shortest incubation times (sugar monophosphates, phosphogluconate, phosphoenolpyruvate, and ribose monophosphate) indicating that these substances play an early role in glucose metabolism. As time progressed a peak of the percentage of the radio-activity incorporated passed through the sugar diphosphates, phosphoglyceric acid, and phosphoenolpyruvic acid, with activity later accumulating in citrate, glutamate and malate (Table 1). Later, synthetic products took on an increasingly greater significance as activity appeared in ever greater % amounts in the amino acids and nucleotides, and a corresponding fall occurred in the early intermediates (i.e. sugar phosphates). UDPG contained ^{14}C even after 6 sec.; after 61 sec. label appeared in maltose phosphate, and in free maltose after 90 sec., suggesting a biosynthetic route for maltose via UDPG and maltose phosphate. Thirty minutes after the addition of the [^{14}C]glucose almost all the activity had disappeared from the sugar phosphates (which contained over 75 % of the total activity after 6 sec.) and appeared in nucleotides, amino acids and organic acids, with a small amount (1·2 %) in maltose. The rapidity with which ^{14}C entered the nucleotides (28 % of the total activity after 30 min.) is significant, and it will be shown later that nucleotide synthesis is one of the metabolic activities most sensitive to starvation and to metabolic inhibition.

Kinetic studies with starved cells

Earlier work with starved cells had shown that when these cells were supplied with glucose a lag period of 2–3 hr. was apparent before the rate of oxidation became maximal (Moses, 1954), and that the lag applied not only to the rate of oxygen uptake but also to the rate of glucose utilization (Moses, 1955*a*). Bartlett & Moses (1957) were able to confirm that [^{14}C]glucose was incorporated into various cellular components, insoluble as well as soluble, more slowly by starved cells than by freshly harvested hyphae. They also found considerable differences in metabolic patterns as between starved and unstarved cells, but were unable to identify many of the compounds which showed most of the differences.

In the present investigation a kinetic study of the incorporation of ^{14}C from [^{14}C]glucose was made by using incubation times between 8 sec. and 30 min.; the patterns at seven of these times are given in Table 2. Compared with unstarved cells (Table 1) several differences are apparent. Activity remained at a much higher degree in the sugar phosphates, still being 23 % of the total after 30 min., as compared with 3·5 % in unstarved cells. The processes of metabolism appeared to have been slowed down: in starved cells radioactivity did not appear in phosphogluconate until 31 sec. (6 sec. in fresh cells), ribose monophosphate never incorporated as much activity as it did in unstarved cells, and % activity did not reach a peak in the sugar diphosphates until 31 sec. as compared with 8–10 sec. for unstarved cells. This agrees with the earlier findings (Bartlett & Moses, 1957) that the oxidative pathway played a less prominent part in glucose metabolism following starvation.

The most important differences, however, appeared in the quantitative distribution of activity in substances formed after a longer incubation period (30 min.). Considerable activity still remained in the sugar phosphates, lesser amounts of labelled amino acids, organic acids and nucleotides were formed (25–50 % of the amounts in fresh cells), and enormous amounts of ^{14}C were diverted into maltose, so much so that some 30 % of the total ^{14}C was present in this sugar. At an earlier stage, free fructose also appeared.

Although with the present techniques it was not possible to compare the absolute rates of [^{14}C]glucose utilization in fresh and starved cells, an earlier study showed that when the same batch of cells was used as the source of both fresh and starved material, the latter were less active than the former (Bartlett & Moses, 1957).

Effects of ammonia and azide on glucose metabolism

It was reported by Moses (1954) that glucose oxidation in starved cells was greatly stimulated by small amounts of ammonia, a stimulation which could be totally inhibited by sodium azide. Azide prevented the incorporation of ammonia-nitrogen by the cells and also increased both the endogenous respiration and that percentage of the glucose utilized which was completely oxidized to carbon dioxide and water. It therefore appeared to be of interest to study the effects of ammonia and azide, alone and together, on the patterns of glucose metabolism.

Fresh unstarved cells were, as might be expected, not short of nitrogen and showed little or no response to the presence of 2×10^{-3}M-ammonium chloride; the incorporation pattern of ^{14}C from [^{14}C]glucose showed little or no difference when ammonia was added to fresh cells.

With starved cells there was a marked effect due to 2×10^{-3}M-ammonium chloride added 2 min. before the [^{14}C]glucose. The % incorporation into maltose, the amino acids, and the organic acids was virtually unaffected, but there

Table 2. *Incorporation of* ^{14}C *from* [^{14}C]*glucose by starved* Zygorhynchus moelleri

Starved cells were supplied with labelled glucose; experimental conditions as in Table 1. The values given are expressed as the % of the total ^{14}C incorporated into all soluble compounds from labelled glucose which is present in each individual substance. The ^{14}C remaining in the residual glucose was excluded from all calculations.

	Incubation period (sec.)						
	9	31	60·5	90·5	122	149	1800*
Sugar diphosphates†	4·2	5·8	4·2	2·8	2·0	3·3	1·1
Sugar monophosphates‡	85·2	61·8	76·5	74·9	63·3	61·3	19·9
Phosphoglycerate	1·2	1·2	0·87	0·90	1·1	1·7	0·91
Phosphoenolpyruvate	0·31	0·31	0·65	0·43	0·52	0·63	0·24
Phosphogluconate	0·0	0·54	0·55	0·60	0·56	0·50	0·0
Ribose monophosphate	2·0	1·4	1·5	1·3	0·88	1·2	0·58
Glycerol phosphate	1·3	3·0	0·74	0·93	0·52	1·4	0·69
Maltose phosphate	0·0	0·0	0·41	1·1	5·5	3·9	0·0
Uridinediphosphoglucose	1·5	5·8	4·5	4·8	7·6	6·0	2·0
Uridinediphosphoribose	0·0	0·0	0·43	0·62	0·51	0·08	2·2
Nucleotide diphosphates§	0·0	0·34	0·65	0·72	1·3	1·3	11·4
Guanosine monophosphate	0·0	0·0	0·0	0·0	0·0	0·0	1·1
Uridine triphosphate	0·0	0·0	0·0	0·0	0·0	0·08	0·85
Adenosine diphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·96
Adenosine triphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·16
Unidentified nucleotide monophosphate	0·0	1·4	1·8	1·9	1·7	1·5	0·0
Maltose	0·0	0·0	0·74	0·57	4·3	2·9	30·6
Fructose	4·3	12·2	4·2	5·2	6·5	7·8	0·0
Succinate	0·0	0·0	0·0	0·0	0·0	0·0	0·12
Fumarate	0·0	0·0	0·0	0·0	0·0	0·0	0·27
Citrate	0·0	0·0	0·05	0·10	0·05	0·13	1·4
Malate	0·0	0·0	0·0	0·03	0·01	0·12	1·9
Glycerate	0·0	0·0	0·09	0·10	0·20	0·22	0·0
Aspartate	0·0	0·0	0·47	0·70	0·75	0·96	3·7
Glutamate	0·0	0·0	0·0	0·20	0·10	0·21	10·8
Alanine	0·0	4·2	1·1	1·4	1·9	3·7	5·7
Glutamine	0·0	0·0	0·0	0·0	0·0	0·0	2·1
Valine	0·0	2·0	0·33	0·43	0·62	0·85	0·33
Histidine	0·0	0·0	0·0	0·0	0·0	0·0	0·33
Tyrosine	0·0	0·0	0·05	0·10	0·10	0·13	0·15
Citrulline	0·0	0·0	0·0	0·0	0·0	0·0	0·33
Unknown substances	0·0	0·0	0·12	0·20	0·0	0·12	1·4
Total in sugar phosphates	94·2	74·1	85·4	83·0	74·4	73·9	23·4
Total in uridine sugars	1·5	5·8	4·9	5·4	8·1	6·1	4·2
Total in nucleotides	0·0	1·7	2·5	2·6	3·0	2·9	14·5
Total in free sugars	4·3	12·2	4·9	5·8	10·8	10·7	30·6
Total in organic acids	0·0	0·0	0·1	0·2	0·3	0·5	3·7
Total in amino acids	0·0	6·2	2·0	2·8	3·5	6·7	23·4
Total activity/100 μl. cells (dis./min. $\times 10^{-5}$)	2·52	8·15	16·94	23·98	31·88	39·24	24·25

* Separate experiment. † Contains diphosphates of fructose, glucose, and glyceric acid. ‡ Contains monophosphates of glucose, fructose, and sedoheptulose. § Contains diphosphates of uridine, inosine, and guanosine.

was a noticeable decrease in the % activity in the sugar phosphates and a corresponding rise in the nucleotides (Table 3). The addition of azide (10^{-3}M) 10–15 min. before the [^{14}C]glucose resulted in the almost total inhibition of both nucleotide and maltose production, a decrease in the total activity incorporated, and of the % activity in the sugar phosphates, and a rise in the ^{14}C in the amino acids and organic acids (Table 3). The presence of azide + ammonia resulted in a picture very similar to that with azide alone. There was a slight rise in the sugar phosphates and a corresponding fall in the organic acids, and some redistribution of activity between aspartate, glutamate and alanine, but otherwise the pictures presented when azide was present, with and without ammonia, were almost identical (Table 3).

The effects of azide thus appeared to be directed mainly towards energy-requiring processes: synthesis of disaccharides (maltose) and nucleotides. Protein synthesis was apparently also impaired: Moses (1954) showed that the incorporation of ammonia nitrogen into the insoluble nitrogen of the cells (i.e. protein) was blocked by azide. The high incorporation of ^{14}C into amino acids and organic acids might then result from a blocking of protein synthesis with a subsequent accumulation of the raw materials for the manufacture of proteins, the amino acids. This resulted, in turn, in a backing-up of the organic acid precursors of the amino acids.

The well-known uncoupling of respiration and oxidative phosphorylation by azide (Simon, 1953), and the consequent increased and probably uncontrolled endogenous respiration of the cells, would also account for the lowered activity found in the sugar phosphates when azide was added to the cell suspensions.

Typical radio-autograms, showing the chromatographic relations of the substances investigated, and the advantages of long-time chromatography for separating the substances in the phosphate area, are shown in Pl. 1, figs. 1 and 2.

DISCUSSION

The path of glucose metabolism in *Zygorhynchus moelleri* which emerges from the present investigations is entirely consistent with earlier studies to elucidate the routes of glucose breakdown in biological systems. The rapid formation from free glucose of glucose and fructose monophosphates, of fructose diphosphate and small quantities of glucose diphosphate, of phosphoglyceric acid, phosphoenolpyruvic acid, together with traces of glyceric acid in the diphosphate area (suggesting the presence of diphosphoglyceric acid), points to an involvement of the glycolysis pathway. This is supported by the appearance of ^{14}C in glycerol phosphate (presumably from triose phosphate) and in alanine and valine, probably from pyruvate. Similarly, a number of intermediates of the oxidative pathway have been detected: phosphogluconic acid, ribose monophosphate and sedoheptulose monophosphate. These compounds, too, appeared after only a few seconds of incubation with [^{14}C]glucose.

At a later stage substances associated with the tricarboxylic acid cycle appeared, and this cycle has earlier been shown to play a role in the respiratory

Table 3. *Effect of ammonium chloride and sodium azide on the incorporation of* ^{14}C *from* $[^{14}C]$*glucose by starved* Zygorhynchus moelleri

Starved cells were supplied with labelled glucose; experimental conditions as in Table 1. The values given are expressed as the % of the total ^{14}C incorporated into all soluble substances, which is present in each individual compound. The ^{14}C remaining in the residual glucose was excluded from all calculations. Concn. of added substances: NH_4Cl, 2×10^{-3} M; NaN_3, 10^{-3} M.

	Plus NH_4Cl		Plus NaN_3		Plus NH_4Cl and NaN_3	
Incubation period (sec.) ...	180	1800	180	1800	180	1800
Sugar diphosphates*	1·2	0·22	0·82	0·36	0·99	0·35
Sugar monophosphates†	39·3	6·3	9·3	3·0	9·1	3·0
Phosphoglycerate	3·1	1·8	12·2	2·7	10·6	6·6
Phosphoenolpyruvate	0·76	0·42	2·9	0·62	2·9	1·7
Phosphogluconate	‡	0·08	0·94	0·08	0·82	0·39
Ribose monophosphate	1·1	0·23	1·0	0·20	0·59	0·13
Glycerol phosphate	3·3	0·65	0·0	0·0	0·0	0·0
Maltose phosphate	0·93	0·47	0·0	0·08	0·0	0·07
Uridinediphosphoglucose	11·0	3·0	1·2	1·5	1·0	1·8
Uridinediphosphoribose	4·5	1·5	0·0	0·0	0·0	0·0
Nucleotide diphosphates§	3·6	20·2	0·23	1·1	0·19	1·2
Guanosine monophosphate	2·7	0·97	0·0	0·0	0·0	0·0
Uridine triphosphate	1·5	1·4	0·0	0·06	0·0	0·0
Unidentified nucleotide monophosphate	0·18	0·91	0·0	0·05	0·0	0·05
Maltose	7·6	34·4	3·1	2·3	3·0	1·0
Fructose	4·0	0·43	0·0	0·62	2·2	0·97
Succinate	0·0	0·0	0·0	1·4	0·45	0·81
Fumarate	0·1	0·19	0·42	0·0	0·0	0·48
Citrate	1·8	2·7	14·7	11·0	12·7	3·6
Malate	0·82	0·70	1·7	3·8	2·1	3·7
Glycerate	0·91	0·63	1·4	0·0	3·2	1·2
Aspartate	3·0	6·0	9·1	19·3	12·3	28·5
Glutamate	1·1	6·1	9·7	21·8	7·3	22·4
Alanine	5·7	5·2	26·1	24·3	25·8	15·2
Glutamine	0·0	1·9	0·56	0·82	0·0	1·0
Citrulline	0·0	0·29	0·0	0·31	0·0	0·40
Valine	1·1	0·73	0·75	2·2	1·7	1·4
Proline	0·0	0·14	0·0	0·0	0·0	0·0
Histidine	0·0	0·26	0·0	0·0	0·0	0·0
Tyrosine	0·0	0·35	0·0	0·0	0·0	0·0
Unknown substances	0·80	1·6	3·8	2·4	2·9	3·8
Total in sugar phosphates	49·7	10·2	27·2	7·0	25·0	12·2
Total in uridine sugars	15·5	4·5	1·2	1·5	1·0	1·8
Total in nucleotides	8·0	23·5	0·23	1·2	0·20	1·3
Total in free sugars	11·6	34·8	3·1	2·9	5·2	2·0
Total in organic acids	3·6	4·2	18·2	16·2	18·5	10·0
Total in amino acids	10·9	21·0	46·2	68·7	47·1	68·9
Total activity/100 μl. cells (dis./min. $\times 10^{-5}$)	68·10	64·07	10·24	47·76	14·51	51·79

* Contains diphosphates of fructose, glucose, and glyceric acid. † Contains monophosphates of glucose, fructose and sedoheptulose. ‡ Did not separate on chromatogram. § Contains diphosphates of uridine, inosine, and guanosine.

metabolism of this mould (Moses, 1955*b*; 1957). The uridinediphosphate derivatives of glucose and ribose incorporated activity very early, with the later appearance of maltose phosphate and free maltose. Various nucleotides began to become prominent after about 1 min. of incubation, and increased to such an extent that after 30 min. with unstarved cells more activity was found in the nucleotides than in any other group of compounds except the amino acids.

The main differences to be seen between fresh and starved cells are those to be expected from nitrogen and carbohydrate starvation: a rapid production of maltose, probably later converted into insoluble polysaccharide reserve materials, and a decreased formation of amino acids and nucleotides by the starved cells. Furthermore, a smaller % of the total ^{14}C incorporated in starved cells appeared in intermediates of the oxidative pathway; this agrees with a previous estimate of the role of the oxidative versus the glycolytic pathways in these cells under various physiological conditions (Bartlett & Moses, 1957).

Ammonia had little effect on the incorporation patterns of unstarved cells and, in starved cells, increased mainly the % of the total ^{14}C incorporated into nucleotides, with a corresponding fall in the % activity in the sugar phosphates. There was no increase in the % of ^{14}C found in the amino acids and the production of maltose remained at a high level. It was the synthesis of nucleotides, together with that of maltose, which was mainly affected by azide, which did not appear to inhibit the formation of amino acids. When azide was present the total amount of ^{14}C incorporated into the soluble fraction of starved cells was increased after 30 min. of incubation. Both the absolute amounts and the % of ^{14}C found in the organic and amino acids increased very greatly in the presence of azide, while the ^{14}C observed in the sugar phosphates, nucleotides and maltose showed a marked fall on both an absolute and a percentage basis.

Ammonia had only a slight effect in the presence of azide, and this concerned mainly the relative distribution of activity in glutamate, aspartate and alanine; the inhibition of ammonia uptake by azide (Moses, 1954) was reflected by the lack of influence of this substance on the ^{14}C-incorporation pattern when azide was present.

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REFERENCES

Bartlett, G. W. & Moses, V. (1957). The pathway of glucose metabolism in *Zygorrhynchus moelleri*. *J. gen. Microbiol.* **16**, 550.

Bassham, J. A. & Calvin, M. (1957). *The Path of Carbon in Photosynthesis.* Englewood Cliffs, N.J.: Prentice-Hall, Inc.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. *J. Amer. chem. Soc.* **72**, 1710.

Calvin, M. (1956). The photosynthetic carbon cycle. *J. chem. Soc.* 1895.

Claridge, C. A. & Werkman, C. H. (1954). Intermediates of the aerobic dissimilation of 2-ketogluconate by *Pseudomonas aeruginosa*. *Arch. Biochem. Biophys.* **51**, 395.

Cohen, S. S. (1953). Studies on D-ribulose and its enzymatic conversion to D-arabinose. *J. biol. Chem.* **201**, 71.

Goldschmidt, E. P., Yall, I. & Koffler, H. (1956). Biochemistry of filamentous fungi. IV. The significance of the tricarboxylic acid cycle in the oxidation of acetate by *Penicillium chrysogenum*. *J. Bact.* **72**, 436.

Heath, E. C. & Koffler, H. (1956). Biochemistry of filamentous fungi. II. The quantitative significance of an 'oxidative pathway' during the growth of *Penicillium chrysogenum*. *J. Bact.* **71**, 174.

Klevstrand, R. & Nordal, A. (1950). A spraying reagent for paper chromatograms which is apparently specific for ketoheptoses. *Acta chem. scand.* **4**, 1320.

Milhaud, G. & Aubert, J. P. (1955). Étude cinétique du métabolisme du glucose et de l'éthanol radioactifs par la levure de boulangerie. *C.R. Acad. Sci., Paris*, **241**, 525.

Moses, V. (1954). The effect of ammonia on the oxidation of glucose by *Zygorrhynchus moelleri*. *Biochem. J.* **57**, 547.

Moses, V. (1955*a*). Glucose respiration in *Zygorrhynchus moelleri*; the entry of glucose into the cells. *J. exp. Bot.* **6**, 222.

Moses, V. (1955*b*). Tricarboxylic acid cycle reactions in the fungus *Zygorrhynchus moelleri*. *J. gen. Microbiol.* **13**, 235.

Moses, V. (1957). The metabolic significance of the citric acid cycle in the growth of the fungus *Zygorrhynchus moelleri*. *J. gen. Microbiol.* **16**, 534.

Moses, V. & Calvin, M. (1958). The path of carbon in photosynthesis. XXII. The identification of carboxy-ketopentitol diphosphates as products of photosynthesis. *Proc. nat. Acad. Sci., Wash.* **44**, 260.

Putman, E. W. & Hassid, W. Z. (1952). Isolation and purification of radioactive sugars by means of paper chromatography. *J. biol. Chem.* **196**, 749.

Simon, E. W. (1953). The action of nitrophenols on respiration and on glucose assimilation in yeast. *J. exp. Bot.* **4**, 377.

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). Detection of sugar on paper chromatograms. *Nature, Lond.* **166**, 444.

EXPLANATION OF PLATE

Fig. 1. Radio-autogram of chromatogram of extract of unstarved cells supplied with [^{14}C] glucose for 30 min.

Fig. 2. Radio-autogram of chromatogram of extract of unstarved cells supplied with [^{14}C]glucose for 3 min. Chromatogram developed with both solvents for a long period in order to spread the phosphate area.

Key: UK, unknown; PEP, phosphoenolpyruvate; PGA, phosphoglycerate; P-gluconate, phosphogluconate; Mono-P, monophosphate; Di-P, diphosphate; UDPG, uridinediphosphoglucose; UDPR, uridinediphosphoribose; UTP, uridine triphosphate; FMP, fructose monophosphate; SeMP, sedoheptulose monophosphate; GMP, glucose monophosphate.

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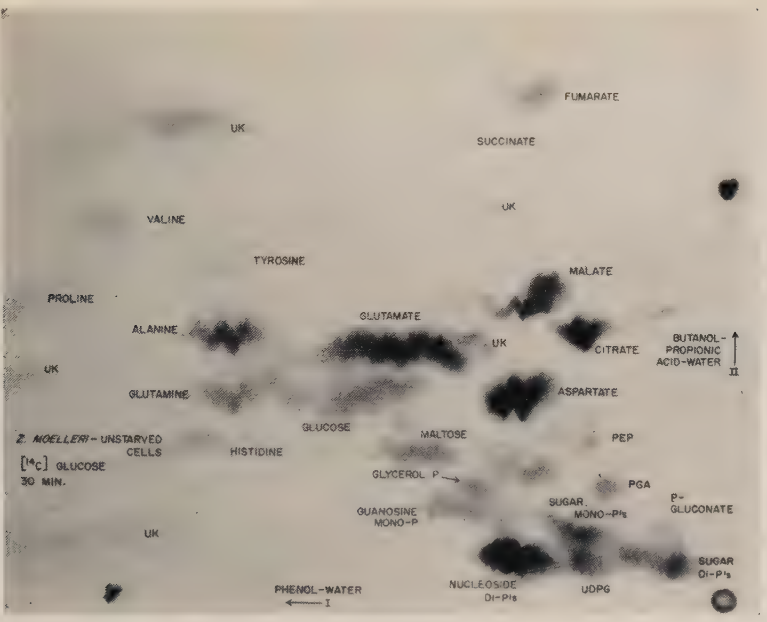

Fig. 1

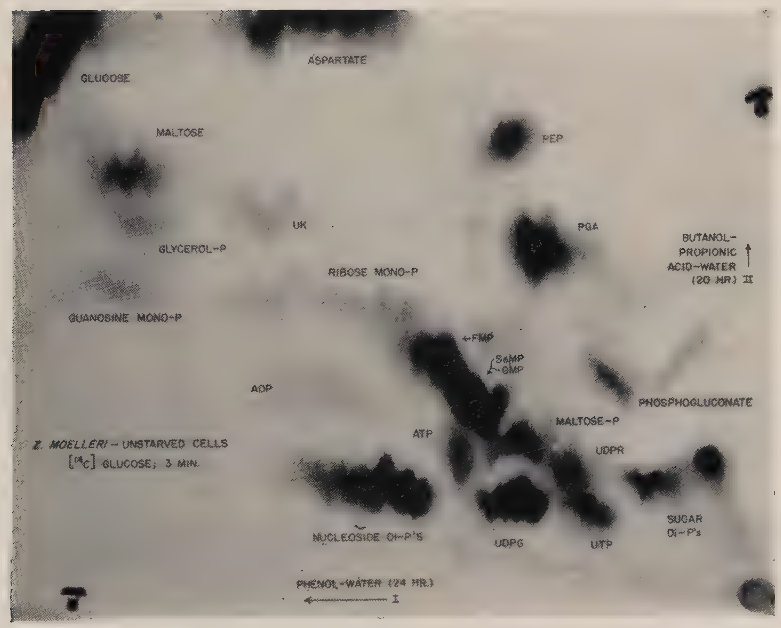

Fig. 2

V. MOSES—[^{14}C]GLUCOSE METABOLISM IN FUNGAL CELLS. PLATE 1

(Facing p. 196)

Kotyk, A. & Kleinzeller, A. (1958). *J. gen. Microbiol.* **20**, 197–212

Movement of Sodium and Cell Volume Changes in a Sodium-Rich Yeast

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SUMMARY: Sodium-rich yeast was prepared by aerating a yeast suspension in M-NaCl for a period of 60 min. This yeast contained then on the average 0·070–0·075 m-equiv. Na/g. dry wt. On incubating it with glucose, xylose or lactose in water, 0·1 M-KCl or 0·1 M-NaCl it lost some of its sodium, in the last case against an apparent concentration gradient. It appears that this movement of Na ion is of a diffusion character as evidenced by the effect of temperature, by incubation without metabolizable substrates and by the use of metabolic inhibitors; however, the possibility of an active contribution to this efflux cannot be excluded. The movement of Na^+ out of the cell was shown to be connected with considerable changes of cell volume effected above all by changes of osmotic pressure of the medium, sodium being lost as a 0·06 M–0·10 M solution. A discussion of these phenomena is presented.

Conway & Moore (1954) described ionic movements between yeast cells enriched with sodium and their environment, with emphasis laid on the mechanism of transport for Na and K ions. It was shown by Conway, Ryan & Carton (1954) that sodium ions were lost by such yeast into a medium containing either K ion or no electrolyte, while no net loss of Na ion was observed on incubating the yeast in 0·1 M-NaCl without substrate. It has been shown elsewhere (Kotyk, 1958) that potassium affects phosphorylation processes in yeast cells as evidenced by a rise in the steady-state concentration of inorganic phosphate and by an increased formation of metaphosphate, and that this effect of potassium was more pronounced when the yeast suspension had been aerated for *c.* 60 min. in M-NaCl before the incubation.

In the course of the present experiments the content of Na and K in the cells as well as their changes were measured. It was then found that such sodium-rich yeast (hereafter to be designated Na-yeast) lost Na ions when incubated with glucose, xylose or lactose in distilled water, in solutions of KCl, and even in 0·1 M-NaCl when the external concentration of Na ions was supposed to be higher than that inside the cells. As this phenomenon persisted under conditions when no active transport is assumed to take place (non-metabolizable substrates, 0°) a measurement of volume changes of yeast cells was carried out. It was later extended to other conditions of incubation, e.g. under different conditions of aerobiosis, different osmotic pressures of the medium used for washing the yeast pellet and in the presence of several metabolic inhibitors. It was found, in agreement with Conway & Downey (1950), that yeast cells showed volume changes due to different osmotic pressures of the medium which, however, might possibly be affected by metabolic processes of the cell. Details of these experiments will be described here.

Evidence will also be presented that the apparent up-hill transport of sodium observed in the Na-yeast may be explained by the considerable volume changes of the cells.

METHODS

Yeast. In all the experiments described here commercial baker's yeast was used, the properties of which appeared to be satisfactorily normal. It was ascertained several times that its properties corresponded with those of a pure strain of *Saccharomyces cerevisiae* (R12). When fresh, the baker's yeast used here contained 28–30 % (w/w) dry matter, 0·011–0·023 m-equiv. Na/g. dry wt. and 0·570–0·615 m-equiv. K/g. dry wt. Occasionally it was ascertained that the yeast contained no apparent contamination by other types of yeast or by bacteria.

Preparation of the yeast and incubation procedure. The yeast was aerated before an experiment as a 10 % (w/v) suspension in M-NaCl for 20–120 min., then centrifuged and left standing for 2 hr. as a pellet. This period appeared necessary for the attainment of a steady state as evidenced by the values of inorganic phosphate of the organisms (not described in detail here). The pellet was then twice washed in distilled water on the centrifuge and resuspended in the incubation medium used for the specific experiments. The suspension was then placed in a cuvette with a round bottom; a glass tube (a pipette proved to be quite satisfactory) reached near the bottom and served to pass either air or nitrogen into the suspension. Nitrogen was always purified by passing it through two vessels containing freshly prepared alkaline pyrogallol. The cuvette was then placed into a constant-temperature bath when the length of the experiment showed it to be necessary. For shorter experiments, when no kinetic measurements were undertaken, the laboratory temperature (20°–21°) was used and a water bath not used.

Sampling. Two methods were used for stopping further metabolic reactions. The first was that of Werkheiser & Bartley (1957) which uses a silicone oil layer to separate the suspension from the denaturing substance, in our case 10 % trichloroacetic acid. Here the acid was pipetted first, then covered with a layer of silicone H-oil (sp.gr. = 1·05) and finally 1 ml. water was placed carefully on top. Then a sample (1 ml.) of the suspension was mixed with the uppermost layer and quickly centrifuged. For a majority of experiments this method of separating of yeast cells from the suspending medium proved satisfactory. However, when inhibitors such as iodoacetic acid and 2, 4-dinitrophenol were used in a NaCl medium a sudden decrease in Na content was found for which no explanation can be suggested, save that these inhibitors increased the efficiency with which the silicone oil deprived the cell surface of adsorbed ions. Because of this difficulty as well as those described by Werkheiser & Bartley (1957) another method of taking samples was preferred. In this method, used by most workers (e.g. Conway & Moore, 1954), a 3 ml. sample of yeast suspension was rapidly centrifuged at a temperature less than 5°, washed with 3 ml. ice-cold distilled water and again quickly centrifuged. It was found that virtually no sodium or potassium was lost from the cells by this procedure, while the intercellular space was practically devoid of these ions. Both of these methods, however, have the disadvantage of not permitting samples to be taken at very short intervals and thus the kinetic studies of ion movement are only incomplete.

Estimation of sodium and potassium. To estimate the Na and K content of the yeast the centrifuged pellet was dissolved in 65 % perchloric acid, placed in a boiling-water bath for 20 min., the volume adjusted to 10 ml. with redistilled water and filtered. The actual estimation was carried out on a Zeiss single-cell flame photometer or on a laboratory-built two-cell flame photometer, with Li as internal standard. Because of the effect of the specific gravity and viscosity of the measured sample on the flow through the atomizer tube, and thus on the amount of liquid dispersed in the flame, care was always taken that the standard solutions had at least approximately the same specific gravity and viscosity as the measured samples by adding equal proportions of perchloric acid to both. The results are expressed in m-equiv./g. dry wt. unless otherwise stated. All measurements reported here were carried out in duplicate or triplicate in each experiment, the values obtained agreeing within ±3 %.

Determination of cell volume. The cell volume at different stages of the experiment was determined by filling a haematocrit tube with the suspension, cooling it to *c.* 0° to prevent carbon dioxide evolution, and centrifuging for 10 min. at approximately 900 ***g***. Longer centrifugation was avoided because the further packing then obtained was proportional in all cases; moreover, there would then be a risk of carbon dioxide production. The tubes were individually calibrated. 'Volume of cells' is expressed in ml./100 ml. suspension, the values being obtained by subtracting the inulin space (determined essentially as described by Conway & Downey, 1950) from the direct haematocrit reading. The value of this space varied between 21–24 % of the pellet volume. The most frequent value of 22 %, which is in agreement with the results of the above authors, was used. It was not apparently affected by the decrease in cell volume. The results of duplicate estimations agreed within ±1 %.

Measurement of pH value. The pH value was measured by a glass electrode on a zero-point pH meter accurate to 0·01 pH unit.

A statistical evaluation of the results of each complete series of experiments was made, their number being far higher than required for statistical significance.

RESULTS

Changes of sodium and potassium content and of cell volume during aeration of yeast suspension in M-NaCl

Unlike Conway & Moore (1954), who used repeated incubation in sodium citrate with glucose in order to increase the sodium content of yeast cells, a simple aeration in M-NaCl was used here and gave similar results. This procedure has the advantage of simultaneously impoverishing the yeast of its endogenous substrate; such a yeast suspension then responds more readily to a glucose addition. Fig. 1 shows the changes of Na and K content of yeast cells after prolonged aeration in M-NaCl as well as the changes in total cell volume. It follows from the results shown in Fig. 1 that the loss of potassium was roughly compensated by the uptake of sodium, while the sharp decrease in volume was obviously due to the hypertonic external medium and probably had no connexion with the ionic shifts examined here.

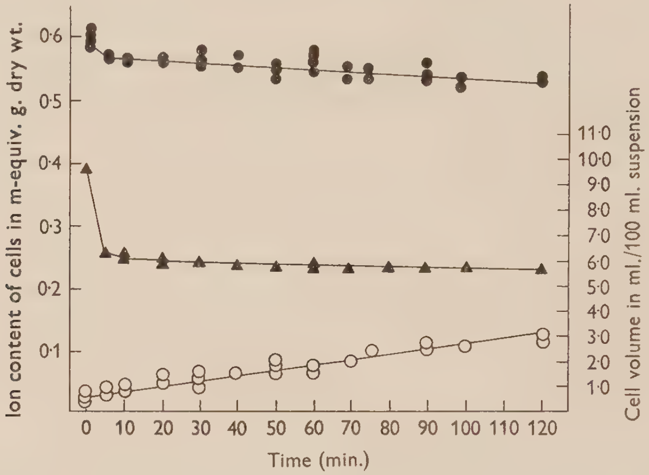

Fig. 1. Na and K content and volume of yeast cells during aeration in 1 M-NaCl. 10 % (w/v) suspension of baker's yeast was aerated in M-NaCl, centrifuged, the pellet left standing for 2 hr., twice washed in ten times its volume of distilled water and resuspended in distilled water. Then Na and K content was estimated. Volume was determined in samples of aerated suspension directly, using individually calibrated haematocrit tubes. ●, K content; ○, Na content; ▲, cell volume.

It is known that the yeast cells contain approximately twice their dry weight as intracellular water (White, 1954, gives the following figures: 65·4 % water, 34·6 % dry matter). In our calculations the 29 % value for dry matter will be used as it was determined repeatedly. If we postulate, for the sake of simplicity, that the 'dry matter' of yeast represents one-third of the total cell volume at the outset of the experiment, i.e. say 3 out of 9 volume units, the solution represents 6 volume units. When the total volume is decreased in the course of aeration by 3·3 volume units, i.e. about 37 %, the 'cell water' volume is decreased by 55 %, assuming that the dry-matter volume does not change. Thus if the Na content of cells in this procedure is increased approximately twice (cf. Fig. 1 at 20 min. aeration) its concentration in the cell water would increase 4·5 times, assuming an even distribution of sodium in the cell water and that none of it was present in the dry-matter space. However, there is no evidence that the sodium taken up is thus evenly distributed; it may well be (as discussed later) that it is more concentrated in a special part of the cell (cf. Conway & Downey, 1950). Its concentration would then increase by the same factor as above. On the other hand, if the total cell volume be considered as contractable and penetrable for Na ions the intracellular sodium concentration would increase 3·2 times under the same conditions.

Changes of Na and K content and of cell volume during incubation of the Na-yeast in various media

Changes of intracellular concentrations of Na and K and of cell volume were studied under different conditions of incubation in order to examine the localization of the sodium taken up by the cell and the rate of its movement out of the cell. For most of these experiments yeast was used which had been previously aerated for 60 min. in M-NaCl and then centrifuged, left standing for 2 hr., washed twice in distilled water and again centrifuged. Its Na content was *c.* 0·070–0·075 m-equiv./g. dry wt. (cf. lower curve in Fig. 1 and subsequent figures, initial values). After washing and resuspending in distilled water the cell volume increased from 5·4 (cf. Fig. 1) to 7·9, which is definitely lower than the original cell volume. Not even a prolonged suspension in water (24 hr.) raised the cell volume to its original value of 9·8.

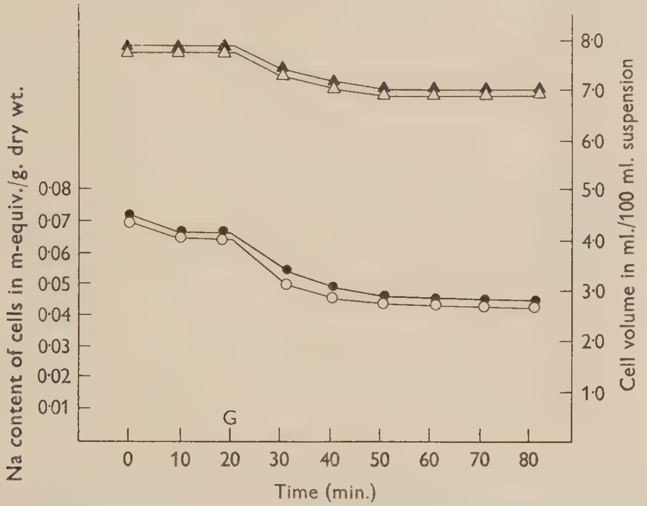

Fig. 2. Na content and volume of Na-yeast cells during incubation in distilled water, aerobically and anaerobically. 10% (w/v) suspension in distilled water, at G glucose added to a final concentration of 0·277 M. ○, Na in air; ●, Na in nitrogen; △, volume in air; ▲, volume in nitrogen.

Incubation in distilled water. When the Na-yeast was suspended in distilled water it gradually lost some of its sodium, especially under aerobic conditions, the cell volume remaining nearly constant. On addition of glucose to final concentration 0·277 M (= 5 %, w/v) a loss of sodium was found both in air and in nitrogen, the aerobic process being somewhat faster. The potassium content of the cells did not change under these conditions.

Fig. 2 shows the average content of Na and volume changes derived from five experiments with incubation in a medium containing no added electrolyte. It can be seen that on addition of glucose the outward movement of sodium down its concentration gradient is accompanied by a decrease in cell volume, the latter being caused probably by an increase of osmotic pressure in the medium due to the addition of glucose. From the results obtained it follows that aerobically sodium left the cells as a 0·07 M-solution. This value was calculated as follows. The cell volume change between 20 and 50 min. of incubation (cf. Fig. 2) was 7·7 − 6·8 = 0·9 ml.; the concomitant change in Na content was 0·064 − 0·042 = 0·022 m-equiv. The concentration of the Na^+ solution leaving the cells would then be (0·022 × 2·9)/0·9 = 0·071 M (2·9 = g. dry matter/100 ml. suspension). Similarly, the concentration figures for incubation in 0·1 M-KCl and 0·1 M-NaCl were derived from Figs. 3 and 4, respectively. As far as the sodium efflux is concerned the Na-yeast used here behaved on incubation in water like the Na-yeast prepared by Conway & Moore (1954; cf. Conway *et al.* 1954).

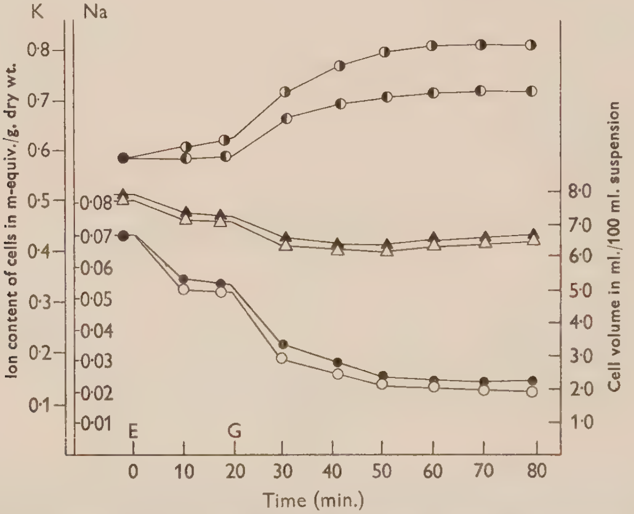

Fig. 3. Na and K content and volume of Na-yeast cells during incubation in 0·1 M-KCl, aerobically and anaerobically. At E KCl was added to a final concentration of 0·1 M, at G glucose to a final concentration of 0·277 M. ○, Na in air; ●, Na in nitrogen; ◑, K in air; ◐, K in nitrogen; △, volume in air; ▲, volume in nitrogen.

Incubation in 0·1 M-KCl. When incubated in KCl solution the Na-yeast behaved much the same as that of Conway & Moore (1954), namely, it lost its sodium rather rapidly, particularly when glucose was present. Fig. 3 shows average values of five such experiments, both under aerobic and anaerobic conditions. It follows from the results obtained that sodium left the cells as a 0·10 M-solution. This value is higher than that calculated for incubation in water, apparently because potassium ions replaced some of the sodium in the cells. A similar decrease in volume was observed upon adding glucose as when incubating in water. Potassium accumulated in the course of this incubation even in the absence of glucose, as found by Rothstein & Enns (1946) with normal yeast.

Incubation in 0·1 M-NaCl. When the Na-yeast was suspended in water and then NaCl solution added to a final concentration of 0·1 M the yeast regularly took up more sodium by a rapid process, both aerobically and generally also anaerobically (see Fig. 4). Simultaneously, a sharp decrease in cell volume of approximately the same magnitude as seen on addition of KCl of the same molarity was observed. When glucose was added to the same final concentration (0·277 M) as in previous experiments, sodium was lost from the cells, both aerobically and anaerobically, while the cell volume decreased further (see Fig. 4), somewhat more than with KCl (average values of eight experiments). The potassium content under the conditions of incubation with glucose in 0·1 M-NaCl did not change at all in *c.* 70 % of the experiments, for the whole duration of an experiment (30–50 min.), while in the rest the potassium content decreased to a varying extent (by at most 0·022 m-equiv./g. dry wt.). There was no difference in the experimental procedure with yeasts giving these two types of response. At any rate, the change in volume was not affected by this movement of potassium.

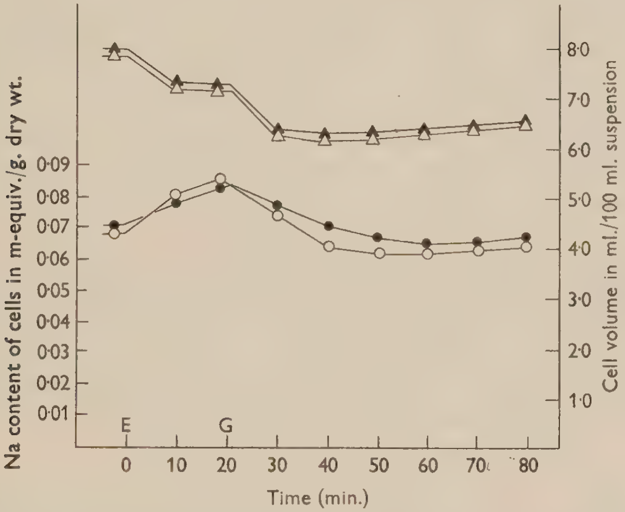

Fig. 4. Na content and volume of Na-yeast cells during incubation in 0·1 M-NaCl, aerobically and anaerobically. 10 % (w/v) suspension in distilled water. At E NaCl was added to a final concentration of 0·1 M, at G glucose to a final concentration of 0·277 M. Symbols as in Fig. 2.

On incubation in 0·1 M-NaCl with glucose, sodium left the cells apparently as a 0·06 M-solution (cf. Fig. 4, between 20 and 50 min.). In other experiments when glucose was added simultaneously with NaCl to the same final concentrations as above, an increase in Na content was followed by a gradual decrease, resembling that shown in Fig. 4; the cell volume also decreased to the same final value as there shown.

The experiments illustrated in Fig. 4 show that without substrate sodium was taken up by the yeast cells with a simultaneous decrease in cell volume. This uptake of Na ion would be most simply explained by the movement of Na according to its concentration gradient (see later). The decrease in volume observed after the addition of electrolytes appears to be due simply to an increased osmotic pressure of the medium and to a consequent movement of water from the yeast cells.

On adding glucose to a final concentration of 0·277 M a further decrease in volume would be expected. Actually, here the volume decrease was gradual and its magnitude was considerably less than would correspond to the molarity of glucose when compared with that of NaCl or KCl. An explanation of this less-marked decrease may be that suggested by Conway & Downey (1950) namely that glucose, by being metabolized, may disappear from the external medium rapidly enough to account for a significant decrease of the osmotic gradient. The volume decrease due to glucose was accompanied by a loss of sodium indicating that the cells did not lose water as such but rather as a solution of NaCl. The apparent intracellular concentration of Na (when only cell water volume is considered, volume changes taken into account, the dry-matter volume assumed to be constant and the sodium ions to be in solution) changed on 30 min. of incubation with 0·277 M-glucose from 0·039 to 0·031 M in distilled water, from 0·037 to 0·019 M in 0·1 M-KCl, and from 0·058 to 0·057 M in 0·1 M-NaCl (cf. figs. 2–4, respectively).

A statistical analysis of paired values showed that the differences in the magnitude of loss of Na after the addition of glucose under aerobic and anaerobic conditions were highly significant (at a $p < 0{\cdot}01$); the differences in volume decrease under aerobic conditions and anaerobic conditions were significant at a level of $p < 0{\cdot}05$ (total of eighteen experiments).

The apparent transport of Na ion against concentration gradient

While the ion movements observed during incubation of Na-yeast in distilled water and in KCl seemed to be in agreement with already published data (Conway *et al.* 1954; Rothstein & Enns, 1946) the sodium loss as observed during an incubation in 0·1 M-NaCl with glucose was unexpected, as it proceeded seemingly against a concentration gradient of Na, the apparent intracellular concentration of Na being about 0·025 m-equiv./ml. whole cell (cf. Fig. 4, at G). In order to test whether this process is to be considered as an active transport several types of experiments were carried out.

(1) Na-yeast was incubated with glucose in 0·1 M-NaCl at 0° and at 30°. Average results of four such experiments (Fig. 5), indicated that the sodium efflux was more or less of a diffusion character as it proceeded (although at a lower rate) even at a temperature at which active transport processes are considered to be practically negligible (Glynn, 1957; Cort & Kleinzeller, 1958).

(2) The changes of cell volume and of Na and K content of cells on addition of 0·277 M-glucose were compared with those resulting from an addition of xylose and lactose to the same molarity. While neither of the latter sugars was metabolized by this yeast, its cell membrane was permeable to xylose, an apparent intracellular equilibrium concentration being reached within 60 min.; on the other hand, lactose did not enter the cells (Burger, Hejmová & Kleinzeller, 1958). It was found (see Table 1) that the decrease in cell volume, accompanied by a loss of sodium from the cells, occurred also with xylose and lactose, although these sugars cannot serve as a source of metabolic energy.

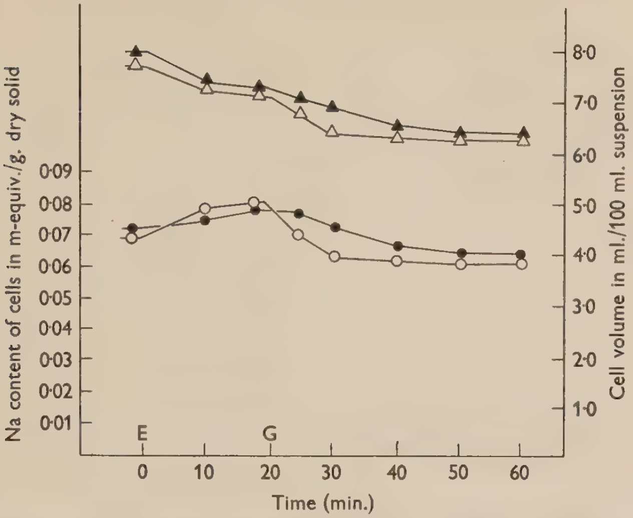

Fig. 5. Na content and volume of Na-yeast cells during incubation in 0·1 M-NaCl, aerobically, at 0° (●, Na; ▲, volume) and at 30° (○, Na; △, volume). At E NaCl was added to a final concentration of 0·1 M, at G glucose to a final concentration of 0·277 M.

Table 1. *Cell volume, Na and K content of Na-yeast cells on incubation with 0·277 M-glucose, xylose and lactose*

Aerobic conditions, averages of three experiments

Sample taken		A			B			C	
	I	II	III	I	II	III	I	II	III
10 min. after suspending in water	7·8	0·070	0·585	7·8	0·069	0·585	7·8	0·070	0·585
10 min. after adding NaCl to 0·1 M (*x*)	7·1	0·083	0·583	7·1	0·083	0·581	7·1	0·048	0·580
1 min. after adding sugar	6·5	0·074	0·580	6·1	0·065	0·580	6·2	0·070	0·582
10 min. after adding sugar	6·2	0·066	0·580	6·0	0·060	0·576	5·8	0·058	0·580
20 min. after adding sugar	6·1	0·064	0·580	6·1	0·063	0·580	5·7	0·056	0·580
30 min. after adding sugar	6·1	0·062	0·576	6·2	0·064	0·576	5·7	0·056	0·576
40 min. after adding sugar	6·2	0·062	0·576	6·5	0·065	0·580	5·7	0·054	0·578
50 min. after adding sugar	6·3	0·064	0·580	6·5	0·065	0·580	5·7	0·054	0·578
60 min. after adding sugar	6·4	0·068	0·580	6·7	0·066	0·580	5·7	0·054	0·576

Addition at (*x*): A, glucose; B, xylose; C, lactose, always to a final concentration of 0·277 M. I, cell volume in ml./100 ml. suspension; II, m-equiv. Na/g. dry wt. III, m-equiv. K/g. dry wt.

The greatest decrease was observed upon addition of lactose, in agreement with the fact that the cellular membrane is impermeable to this sugar, thus preventing an equilibration of the concentrations on both sides of the membrane. Addition of xylose caused the cell volume to decrease as sharply as with lactose; however, the cell volume returned to nearly the initial value in the course of 60 min., thus reflecting the gradual establishment of an osmotic equilibrium. These facts again indicate that the volume decrease was due above all to osmotic conditions in the medium as opposed to those in the cell, and that Na ions were lost as the volume decreased.

(3) $2{\cdot}5 \times 10^{-5}$ M-dinitrophenol, 5×10^{-4} M-iodoacetic acid, and 10^{-3} M-sodium azide were used as metabolic inhibitors to provide further information about the character of the sodium loss. The results of three experiments carried out in 0·1 M-NaCl with 0·277 M-glucose are summarized in Table 2.

The reason for smaller volume changes with dinitrophenol, as compared with the control, may lie in the fact that this inhibitor increased the glucose consumption by reversing the Pasteur effect and therefore offset the volume decrease caused by hypertonic glucose medium. Iodoacetic acid showed no effect on the decrease of the yeast cell volume; but it is of interest to note that potassium was lost from the cells while little sodium disappeared. Sodium azide produced a sharp decrease in volume, but in relation to this a smaller loss of sodium ions than expected from the results obtained in the presence of glucose either alone or with dinitrophenol. However, the very rapid loss of potassium with azide (and to some extent also with iodoacetic acid) might be responsible for this unexpected behaviour.

All the inhibitors used affected to some extent the decrease in cell volume brought about by the addition of glucose, apparently by interfering either with the mechanism of glucose utilization or with ionic movements. These results by themselves are not conclusive evidence for a direct metabolic influence on the contraction processes of the yeast cell.

(4) Glucose was applied at 0·0277 M, which is sufficient to induce metabolic reactions but is relatively unimportant osmotically. The results of this addition, as shown in Table 3 in comparison with 0·277 M-glucose, indicate that no significant loss of sodium and no decrease in cell volume was observed under these conditions. It follows, therefore, that the cell volume and Na content decrease brought about by the addition of glucose is not of a primarily metabolic character.

(5) The results described above can be taken as evidence for the view that the loss of Na in the presence of glucose, xylose or lactose against an apparent concentration gradient as observed with this Na-yeast is essentially of a passive character. Na ions move out of the cells as a 0·06 M-solution (see p. 203) either because of the shrinkage of the cell (assuming that no higher concentration can exist in the cell), or according to a concentration gradient (assuming that Na is present at least in some part of the cell at a higher concentration than that of the medium). In order to test which of these assumptions better expresses the above facts and to determine the actual intracellular concentration of Na, the yeast pellet after aeration in M-NaCl was washed in different concentrations of NaCl ranging from 0 to 0·255 M. The washing was repeated three times, followed by very rapid suspension of the pellet in ice-cold distilled water, during which time no sodium was lost from the cells, as confirmed specially by using ^{24}Na-rich yeast. Table 4 shows that in the course of washing with NaCl solutions between 0·150 and 0·225 M the sodium content of cells was constant, regardless of the Na concentration in the medium, while sodium was lost from the cells at concentrations below 0·150 M. This result, obtained in

Table 2. *Cell volume, Na and K content of cells with dinitrophenol, iodoacetic acid and sodium azide*

Aerobic conditions; averages of three experiments.

Sample taken		A			B			C			D		
		I	II	III	I	II	III	I	II	III	I	II	III
10 min. after suspending in water		7·9	0·070	0·587	7·9	0·070	0·587	7·9	0·071	0·585	7·9	0·070	0·588
10 min. after adding NaCl to 0·1 M		7·1	0·084	0·583	7·1	0·084	0·581	7·1	0·083	0·582	7·1	0·884	0·583
(*x*)													
10 min. after adding inhibitor		7·1	0·084	0·583	7·1	0·083	0·581	7·1	0·080	0·562	7·0	0·084	0·518
1 min.	after adding glucose	6·5	0·074	0·583	6·5	0·075	0·580	6·5	0·071	0·559	6·2	0·070	0·510
10 min.		6·2	0·068	0·583	6·3	0·071	0·585	6·2	0·073	0·549	6·1	0·069	0·424
20 min.		6·1	0·064	0·583	6·2	0·070	0·582	6·1	0·073	0·054	6·1	0·067	0·392
30 min.		6·0	0·062	0·581	6·2	0·067	0·580	6·0	0·074	0·541	6·1	0·065	0·367
40 min.		6·1	0·062	0·579	6·2	0·065	0·581	6·1	0·075	0·532	6·1	0·066	0·347
50 min.		6·2	0·064	0·580	6·2	0·065	0·582	6·2	0·074	0·529	6·0	0·063	0·329
60 min.		6·3	0·066	0·578	6·4	0·067	0·582	6·2	0·075	0·522	6·0	0·066	0·313

Additions at (*x*): A, 0·1 M-NaCl; B, dinitrophenol to $2{\cdot}5 \times 10^{-5}$ M; C, iodoacetic acid to 5×10^{-4} M; D, sodium azide to 10^{-3} M.
I, cell volume in ml./100 ml. of suspension; II, m-equiv. Na/g. dry wt.; III, m-equiv. K/g. dry wt.

three consecutive experiments, might indicate that 0·15 M is the actual maximal Na concentration that exists in some part of the cell. At the same time it is of interest to return to the fact that a subsequent addition of NaCl (at concentrations even below 0·15 M without substrate) to a suspension of yeast previously washed with water brought about an increase in the Na content of

Table 3. *Cell volume and Na content of Na-yeast cells during incubation with various concentrations of glucose*

Aerobic conditions; averages of five experiments.

		A		B		C	
Sample taken		I	II	I	II	I	II
10 min. after suspending in dist. water		7·9	0·070	7·8	0·071	7·9	0·070
10 min. after adding NaCl to 0·1 M		7·1	0·082	7·1	0·082	7·1	0·083
(*x*)							
1 min.	after adding glucose	7·1	0·082	7·1	0·083	6·5	0·077
10 min.		7·1	0·082	7·2	0·084	6·2	0·069
20 min.		7·1	0·081	7·2	0·082	6·2	0·064
30 min.		7·0	0·080	7·1	0·082	6·2	0·065
40 min.		7·0	0·080	7·1	0·082	6·2	0·065
50 min.		6·9	0·080	7·1	0·082	6·2	0·065
60 min.		7·0	0·081	7·2	0·083	6·4	0·066

Addition at (*x*): A, 0·1 M-NaCl; B, glucose to a final concentration of 0·0277 M (in 0·1 M-NaCl): C, glucose to a final concentration of 0·277 M (in 0·1 M-NaCl).

I, cell volume in ml./100 ml. of suspension; II, m-equiv. Na/g. dry wt.

Table 4. *The effect of washing with various concentrations of NaCl on the Na content of Na-yeast cells*

In m-equiv. Na/g. dry wt. after triple washing following 60 min. aeration in 1·0 M-NaCl. Average values of three experiments.

Molarity of NaCl used for washing	Na content	Molarity of NaCl used for washing	Na content
0	0·068	0·025	0·084
0·025	0·070	0·150	0·085
0·050	0·075	0·175	0·085
0·075	0·080	0·200	0·085
0·100	0·082	0·225	0·085

the cells (see Fig. 4). The increased Na content, however, never exceeds the value 0·085 m-equiv./g. dry matter, which is also the maximum Na content remaining in the cells after washing (cf. Table 4, at 0·150 M-NaCl and higher). It is impossible at present to offer an explanation of this effect, but it seems likely that the process of washing in water removes Na ions from peripheral parts of the cells (possibly the Na space of Conway & Downey, 1950) or from the surface, which would then become unsaturated as to Na. The added NaCl would then tend to saturate the surface or peripheral parts, possibly by adsorption. Such a view appears to be of interest because it has been found in further experiments, not described in detail here, that the apparent concentration of

Na in and on the yeast cells after aeration in M-NaCl and subsequent centrifugation (allowing 30% for the intercellular space at 500 *g*) amounted to 0·6 M.

In conclusion, from the above set of experiments it may be said that the osmotic component involved in the movement of sodium ions and water appears to play a major role. The explanation is assumed to be that Na is present in some part of the cell at a concentration higher than in the outside medium, probably at a concentration 0·15 M. There are indications, however, that the volume decrease may also be due to other factors. Besides the effect of inhibitors this is shown by the observation that the cell volume decreased somewhat (Table 5) when air was passed into an anaerobic yeast suspension without substrate; metabolic energy produced in endogenous respiration may be responsible for this volume decrease.

Table 5. *Volume changes of Na-yeast after passing air into an anaerobic yeast suspension*

In ml./100 ml. of suspension; average values of three experiments.

Sample taken	A	B
10 min. after suspending in water	7·9	8·0
	water added	glucose to 0·277 M added
10 min. after adding water (glucose)	8·1	7·2
20 min. after adding water (glucose)	8·3	6·7
10 min. after passing air into the suspension	7·8	6·7
20 min.	7·8	6·7
30 min.	7·9	6·9
40 min.	8·0	6·9
50 min.	8·0	7·0

The observed volume changes appear to have no connexion with the loss of hydrogen ions, as these leave the cells very rapidly when even a relatively small amount of glucose is added (Conway & Brady, 1950) while the volume does not decrease (cf. Table 3). Similarly, the movement of potassium seems to have no general connexion with volume changes.

From microscopical inspection there was no apparent morphological change either in general appearance or in the size of various cell structures before and after addition of glucose.

DISCUSSION

From the results of other authors (e.g. Conway & Downey, 1950) it appears that yeast cells undergo volume changes corresponding to the external osmotic pressure. It has been shown in the present work that this holds true in an even greater degree for the Na-yeast used here. The magnitude of this osmotic adjustment seems to be affected by several factors. Thus it follows from the application of glucose, xylose and lactose, as well as from the comparison of anaerobic and aerobic cell volumes, that the expected decrease in volume due to osmotic pressure can be slowed down and diminished when the substance which causes the increase of osmotic pressure is taken up by the cell from the medium. This might be one explanation of why the cell volume decreases less anaerobically than aerobically, the anaerobic utilization of glucose being higher than the aerobic one. Perhaps for the same reason the decrease in volume with glucose is less in KCl than in NaCl (Rothstein & Enns, 1946), with dinitrophenol than without it, and with glucose than with xylose, or especially with lactose, in the first few minutes after addition. However, metabolic processes also may be involved in the cell volume changes. Thus the difference in cell volume under aerobic and anaerobic conditions (cf. Figs. 2–4) might be explained by metabolic factors, as well as the more rapid decrease in cell volume observed at higher temperatures (cf. Fig. 5). Similarly, the effect of inhibitors seems to be of a more complex nature and some of their effects might be attributed to metabolic influences, e.g. the decrease in cell volume with iodoacetic acid and sodium azide which appears to be associated with a flow of K ion from the cell.

In connexion with the decrease in cell volume it is of interest to note that when yeast has been previously enriched with sodium by a simple aeration in sodium chloride, sodium is lost from the cells against an apparent concentration gradient. It was shown above that the loss of sodium from the cells brought about by the addition of a sugar such as glucose (metabolized), xylose (not metabolized but penetrating into the yeast cell) or lactose (not metabolized and not penetrating into the yeast cell) proceeds under conditions when it can be assumed that no active transport takes place. It was therefore concluded that the loss of sodium associated closely with a decrease in cell volume is of a passive character. Two possible explanations for this loss of sodium offer themselves.

Firstly, if there exists a maximum concentration of sodium in the total cell water which cannot be exceeded, sodium is lost from the cells as a solution when the cell volume decreases. In this way its internal concentration could be maintained if the outgoing solution were of the same concentration as the sodium 'solution' in the cell. The results presented above do not contradict this view: 0·06 M is roughly the concentration of Na ion solution lost by the cells (cf. p. 203) and 0·06 M is about the calculated concentration of sodium in the cell water (cf. p. 204). However, Conway & Moore (1954) reached a far higher concentration of Na^+ in their Na-yeast (as much as 0·2 m-equiv. Na/g. wet yeast).

The second explanation is concerned with the possibility that some or all of the sodium, taken up by the cells in the course of aeration in M-NaCl, is localized in only a part of the cell, where its concentration would be correspondingly higher than the above-mentioned value of 0·06 M. It appears that the results presented above can be better reconciled with such explanation. As shown by experiments (Table 4) the sodium concentration in this part of the cell may be as high as 0·15 M. From this special compartment sodium ions would leak, either according to their concentration gradient or again due to the cell volume decrease if a certain maximum concentration cannot be exceeded. It is difficult to test by which of the last-named ways sodium ions actually leave the cells. In distilled water sodium is lost from the cells obviously by simple diffusion according to its concentration gradient; however, when NaCl solution is used as medium the picture is distorted by the fact that the cell volume decreases at the same time; furthermore, when a more concentrated solution of NaCl is used than that employed for washing the yeast additional sodium is taken up by the cells, possibly by adsorption on the cell surface or by simple diffusion into a peripheral 'sodium' (?) space.

If it be postulated that all the sodium taken up in the course of the preliminary aeration in M-NaCl is localized in the above-mentioned special site in the cell at a concentration of 0·15 M, the size of this site can be estimated in the following way. Yeast takes up, in the course of 60 min. of aeration, on the average 0·065 m-equiv. Na/g. dry wt., i.e. 0·026 m-equiv. Na/ml. total cell volume after aeration and washing. The sodium content of the yeast cell increases further to at most 0·085 m-equiv./g. dry wt. when it is suspended in 0·1 M-NaCl, which represents 0·035 m-equiv. Na/ml. total cell volume at that stage of incubation (cf. Fig. 4 at 20 min.). Therefore, for the concentration of Na inside the cell to reach the value of 0·15 M it must be concentrated in a volume representing about 23 % of total cell volume just before glucose addition. Now, as the cell water volume at this stage is assumed to occupy on the average 59 % of the total volume (the dry-matter volume is considered not to decrease in the course of aeration and incubation; for values see Table 3, column 1) the Na-space would amount to 39 % of the cell water volume. As it is probable that the Na-space decreased in parallel with the decrease of the remaining cell water volume, the Na-space would amount to about 25 % of the total cell volume after aeration and washing and to about 28 % of the total cell volume prior to the aeration. This value is about three times as high as that given by Conway & Downey (1950) for an outer metabolic space of the cell, but is significantly lower than the apparent 'sugar space' found by Burger *et al.* (1958) to represent about 55 % of the original normal yeast cell volume as determined by estimating the amount of a non-metabolized sugar (galactose) inside the cell after equilibration with a galactose-containing medium.

However, there is no way of testing at present whether all the sodium taken up in the course of aeration is localized in the assumed special compartment. If only some part of it is located there the size of this cell space will be smaller and may well approach the value given by Conway & Downey (1950). As a matter of fact by assuming a smaller Na-space freely penetrable by Na ions the partial loss of sodium after washing with water and its additional uptake after adding NaCl to the yeast can be explained.

The suggested explanations of the results obtained may have to be modified when more data are to hand (particularly through the introduction of faster methods of measuring the cell volume and by extending the study to subcellular particles), but they present a point to be considered in drawing conclusions about transport phenomena in yeast cells.

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REFERENCES

BURGER, M., HEJMOVÁ, L. & KLEINZELLER, A. (1959). Transport of some mono- and disaccharides into yeast cells. *Biochem. J.* **71**, 233.

CONWAY, E. J. & BRADY, T. G. (1950). Biological production of acid and alkali. *Biochem. J.* **47**, 360.

CONWAY, E. J. & DOWNEY, M. (1950). An outer metabolic region of the yeast cell. *Biochem. J.* **47**, 347.

CONWAY, E. J. & MOORE, P. T. (1954). A sodium yeast and some of its properties. *Biochem. J.* **57**, 523.

CONWAY, E. J., RYAN, H. & CARTON, E. (1954). Active transport of sodium ions from the yeast cell. *Biochem. J.* **58**, 158.

CORT, J. H. & KLEINZELLER, A. (1958). The effect of temperature on the transport of sodium and potassium by kidney cortex slices. *J. Physiol.* **142**, 208.

GLYNN, I. M. (1957). The ionic permeability of the red cell membrane. *Progr. Biophys.* **8**, 241.

KOTYK, A. (1958). The influence of K^+ on phosphorylation processes in *Saccharomyces cerevisiae*. *Biokhimija*, **23**, 737.

ROTHSTEIN, A. & ENNS, L. H. (1946). The relation of K to carbohydrate metabolism in baker's yeast. *J. cell. comp. Physiol.* **28**, 231.

WERKHEISER, W. C. & BARTLEY, W. (1957). The study of steady state concentrations of internal solutes of mitochondria by rapid centrifugal transfer to a fixation medium. *Biochem. J.* **66**, 79.

WHITE, J. (1954). *Yeast Technology*. London: Chapman and Hall.

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MARTIN, P. G. (1959). *J. gen. Microbiol.* **20**, 213–222

Apparent Self-Fertility in *Neurospora crassa*

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SUMMARY: Single ascospores which gave rise to temporarily self-fertile cultures were found in *Neurospora crassa*. Genetical and cytological evidence suggested that these ascospores were disomic for the chromosome containing the mating-type locus, the disomy having arisen following partial desynapsis at meiosis and unequal distribution of univalents. At some time after germination, the nuclei reverted to the haploid state. If the disomic ascospore had been heterozygous at the mating-type locus, two sorts of haploids might occur and fertility probably resulted from mating between these.

The genes *pdx* (pyridoxineless) and *pdx-p* (pyridoxineless, ammonia-sensitive) in Neurospora are either allelicor pseudo-allelic (Mitchell, 1955). Pseudo-wild type strains (Mitchell, Pittenger & Mitchell, 1952; Pittenger, 1953, 1954) occur among the pyridoxine-independent segregants from the cross *pdx co A* × *pdx-p a*; *co* is a colonial marker gene approximately five units distal to *pdx* and *pdx-p*. Although these grow in the absence of pyridoxine, they segregate approximately 50 % pyridoxineless spores in crosses to wild type. This is thought to result from the spores being disomic for the chromosome carrying the pyridoxineless loci, the disomic nuclei being pyridoxine-independent but not functioning in fertilization; only haploid derivatives of the disomic are fertile (Mitchell *et al.* 1952; Pittenger, 1953, 1954).

Prototrophic segregants from this cross were classified by outcrossing to wild type strains (extracts from Emerson *A* and *a*) and scoring the progeny. Apparent self-fertile strains were observed in the progeny of four out of sixteen of these crosses; in the sequel the abbreviation A.S.F. will be used.

This paper describes the origins of the A.S.F. strains which were investigated and the results of genetical and cytological investigation of them.

METHODS

Strains. The strains of *Neurospora crassa* used were *pdx*: (37803), *pdx-p* (44602); *co* (70007), (Barratt *et al.* 1954). These were descendants of stocks obtained by Professor D. G. Catcheside from Professor G. W. Beadle. The wild type strains used were Emerson *A* and *a* which were extracts from Emerson *A* (5296) × Emerson *a* (5297) and Beadle *a*, an extract from Beadle 25*a* × Beadle 1*A*. The genes *pdx*, *pdx-p* and *co* are not in the same linkage group as the mating-type locus.

Media. The methods used for cleaning glassware, making and sterilizing media were, generally, those recommended by Ryan, Beadle & Tatum (1943). The minimal medium used was that of Westergaard & Mitchell (1947) while, to distinguish *pdx-p* from *pdx*, the 'M/15 phosphate medium' of Strauss

(1951) was used: strain *pdx-p* grows well on the latter while strain *pdx* does not grow at all. Supplements of vitamins and other growth substances were used at the concentrations recommended by Beadle & Tatum (1945).

Methods. Methods of inoculation, making crosses, ascospore isolation from ordered asci and germination and incubation were generally as recommended by Beadle & Tatum (1945). Once the possibility of self-fertility was suspected and exclusion of the possibility of contamination became even more important, care was taken that tubes in which ascospores had been incubated were not opened until after perithecium formation might have been expected. Twenty days were allowed and strains classified as A.S.F. developed perithecia before this time. Where it was important to obtain unequivocal results from mating-type tests, each ascospore isolation was tested against standard strains of both mating types. The strains used were Emerson *A* and Beadle *a*, the latter being preferred to Emerson *a*, which is slow in forming protoperithecia. For positive identification of mating-type, perithecia which contain ascospores in one cross, but not in the other, were required.

Cytology. The techniques used were similar to those described by Singleton (1953) except that acetic acid ethanol was replaced by a fixative described by Newcomer (1953). This appeared to clear the cytoplasm better and gave good fixation of chromosomes. Fixed material was hydrated in stages and then hydrolysed in N-hydrochloric acid at 60° for 6 min. The stain used was 2 % (w/v) synthetic orcein (G. T. Gurr, London) in 60 % (v/v) acetic acid in water. About 6 perithecia were squashed in a drop of stain on an acid-washed slide. A Zeiss microscope fitted with an apochromatic objective (×90, N.A. 1·30) and an aplanatic condenser (N.A. 1·40) was used. Photographs were taken with a 35 mm. Leica camera and Ilford FP3 film.

RESULTS

Origin of A.S.F. strains. In order to classify prototrophs from the cross *pdx co A* × *pdx-p a*, it was necessary to classify their progeny from crosses to wild type strains. Apparent self-fertile strains were observed among the wild type segregants from four of these crosses. The nature of the parents and designation of the A.S.F. strains are shown in Table 1. At first these were attributed to contamination by causes unknown. It was observed, however, that the perithecia contained very few ripe ascospores; but a single ripe ascus was observed and dissected from A.S.F. 1. The spores were germinated in tubes of minimal medium which, after heat treatment, were not opened, thus ensuring freedom from contamination. Spore number 4 did not germinate and of the seven remaining spores six were of mating type A. Spore number 1 produced an A.S.F. strain, henceforth referred to as A.S.F. 9. All seven segregants were pyridoxine-independent, but A.S.F. 9 was subsequently shown to produce *pdx-p* progeny.

This most abnormal result suggested that A.S.F. strain 1 was indeed self-fertile. Accordingly, A.S.F. strains 1–8 were re-examined. Although many perithecia were formed, they contained mostly aborted ascospores. This extreme infertility

Table 1. *Origin and designation of A.S.F. strains*

Prototrophic parent	Other parent	Frequency of A.S.F. strains in progeny	Designated A.S.F. number
Pseudo-wild type	Emerson *A*	4 in 24	1–4
Pyridoxine-independent, non-colonial	Emerson *A*	1 in 19	5
Pyridoxine-independent, colonial	Emerson *A*	1 in 20	6
Pyridoxine-independent, colonial	Emerson *a*	2 in 41	7–8

Table 2. *Segregations observed in asci from A.S.F. strains*

		Segregation of *pdx-p*, *co* and mating type					
		pdx-p		*co*		Mating type	
A.S.F. No.	Progeny	First division	Second division	First division	Second division	First division	Second division
1	1 *pdx-p*:1+	2	0	.	.	1	1
2	1 *pdx-p*:1+	2	1	.	.	2	1
3	All+	.	.	.	.	0	2
5	1 *co*:1+	.	.	1	2	0	3
6	1 *co*:1+	.	.	2	1	2	1
7	All+	.	.	.	.	3	0
8	All +	.	.	.	.	2	2
		4	1	3	3	10	10

suggested that A.S.F. 1–8 all had similar origins and that this was not contamination. A few complete ripe asci were found and dissected with the results shown in Table 2. Since no A.S.F. strains were found among the asci, eighty-three random ascospores from the selfing of A.S.F. 9 were examined and were classified as follows:

	A	*a*	No reaction	Total
pdx-p+	11	15	12	38
pdx-p	15	21	7	43
	26	36	19	81

The segregation ratios of *pdx-p*:*pdx-p*$^+$ and of mating type, where it could be scored, were both approximately 1:1. In addition there were two A.S.F. strains, both pyridoxine-independent, and these have been designated 10 and 11.

All the progeny of A.S.F. 10 and 11 were *pdx-p*$^+$. Four ordered asci out of thirteen from A.S.F. 11 contained new A.S.F. strains designated 12–15 (Table 4).

The properties of A.S.F. strains

(1) *General properties.* Perithecia resulting from selfing first appeared later than expected in an ordinary cross, i.e. when 15–20 days old. In subsequent sub-cultures, however, they appeared after the expected 5 or 6 days. Few ripe ascospores were formed—usually only about 30/perithecium and a completely ripe ascus occurred only in about one in four perithecia. Most ascospores aborted and all combinations of aborted and ripe ascospores were seen. No A.S.F. strain retained its self-fertility for more than two or three subcultures.

(2) *Genetical properties.* In A.S.F. 10, mating type segregated at the second division in ten out of fourteen asci and in A.S.F. 11 in five out of thirteen asci. These figures, combined with the data for A.S.F. 1–8 in Table 2, give a total of twenty-five second-division segregations of mating type in forty-seven asci from selfed A.S.F. strains. The 95 % fiducial limits for this observed proportion are 17·9/47 and 32·1/47 so that the observations differ significantly from the normal expectation of 14 %, i.e. 6·6/47.

Table 3. *Ascus types in progeny of A.S.F. strains*

Parent	Ascus type	Segregation of mating type	Segregation of growth rate	No.
A.S.F. 10	Ditype	First division	First division	9
	Ditype	Second division	Second division	3
	Tetratype	Second division	First division	1
A.S.F. 11*	Ditype	First division	First division	4
	Ditype	Second division	Second division	3
	Tetratype	Second division	First division	3
	Tetratype	Second division	Second division	1

* These eleven asci include nos. 1, 2 and 4 in Table 4.

Asci from A.S.F. 10 and 11 each showed a distinct single-factor segregation for growth rate. However, this may have had different causes in the two cases, for in A.S.F. 10, but not A.S.F. 11, the slow growth rate was accompanied by a fluorescent medium (under u.v. radiation) and the formation of melanin. The factor for growth rate was in both cases linked to mating type and in both cases *A* was usually the faster. Ascus types are shown in Table 3. In both cases it would appear that the gene affecting growth rate had its locus between the centromere and the mating-type locus.

Table 4. *Composition of some abnormal asci from selfing in A.S.F.* 11

Ascus no.	Character	Spore no.							
		1	2	3	4	5	6	7	8
1	Mating type	*a*	A.S.F. 12	*A*	*A*	*A*	*A*	*a*	*a*
	Growth rate	Slow	Slow	Slow	Slow	Fast	Fast	Fast	Fast
2	Mating type	*a*	*a*	*A*	*A*	*A*	*A*	A.S.F. 13	*a*
	Growth rate	Slow	Slow	Fast	Fast	Slow	Slow	Fast	Fast
3	Mating type	Aborted	Aborted	*a*	*a*	*a*	*a*	A.S.F. 14	Aborted
	Growth rate	.	.	Slow	Slow	Slow	Slow	Fast	.
4	Mating type	*a*	*a*	*A*	*A*	*A*	*A*	Aborted	A.S.F. 15
	Growth rate	Fast	Fast	Fast	Fast	Slow	Slow	.	Slow
5	Mating type	*a*	*a*	*a*	*A*	*A*	*A*	*a*	Aborted
	Growth rate	Slow	Slow	Slow	Fast	Fast	Fast	Slow	.

Asci containing, for example, seven mature ascospores and one hyaline aborted spore, suggested that members of a spore pair could differ. This was confirmed in the four asci containing A.S.F. 12–15, and in another ascus, the composition of which, with respect to mating type and the growth-rate factor, are shown in Table 4.

An examination of these asci suggests that A.S.F. strains nos. 12, 13 and 15 should have been *a* while A.S.F. 14 should have been *A*. When these strains reverted to normal they did not necessarily revert to the mating-type expected from position in the ascus. Thus while A.S.F. 13, 14 and 15 reverted to their 'correct' mating types, A.S.F. 12 reverted to *A* instead of *a*. A.S.F. strains 9, 10, and 11 whose 'correct' mating types were uncertain, reverted to *A*, *A* and *a*, respectively. Thus A.S.F. strains revert to a single mating-type, but this is not necessarily that which, from position in the ascus, would be expected; moreover, they seem to revert equally frequently to *A* and *a*.

(3) *Cytological properties.* A complete cytological analysis of self-fertility was not carried out and most observations were confined to the more easily observable stages in the ascus, viz. diakinesis, metaphase I and anaphase I. With orcein staining the spindle and nucleolus were not visible. This, together with the fact that in minute chromosomes it is difficult to distinguish singleness from doubleness, sometimes made unambiguous interpretation of a configuration difficult.

The original cross which gave rise to one parent of the first A.S.F. strains was *pdx A* × *pdx-p a*. From this cross twenty-two asci were observed at diakinesis or metaphase I. In most of these meiosis appeared to be quite normal (e.g. Pl. 1, fig. 1) but in one, eight bodies were present (Pl. 1, fig. 2). These were presumably six bivalents and two univalents. Of ten anaphase I and early telophase I stages, most appeared to be quite normal (e.g. Pl. 1, fig. 3) but in one there appeared to be six bodies at one pole, five at the other, with three laggards (Pl. 1, fig. 4). Presumably five bivalents disjoined normally, leaving three univalents on the equatorial plate while the other univalent was carried to one pole.

If the interpretations of Pl. 1, figs. 2 and 4 are correct, it seems probable that univalents are occasionally formed in this cross. The distribution of these might give rise to aneuploid ascospores which, if disomic for the *pyridoxineless* chromosome, would give rise to the pseudo-wild type strains observed.

A.S.F. strains 1–4 were found in the progeny of the cross of one of these pseudo-wild type strains to Emerson *A*. Among five diakinesis metaphase I configurations and four anaphase I configurations, no abnormalities were detected. However, if univalents were also formed in crosses of this kind and others, an aneuploid ascospore disomic for the mating-type chromosome might give rise to A.S.F. strains.

Plate 1, fig. 5 shows meiosis in an ascus resulting from selfing in A.S.F. strain 11. The stage appears to be early anaphase I, but there are more than the expected seven bodies going to each pole. There appear to be twelve bodies at one pole, nine at the other and one between the two groups. This figure suggests either that the original zygote was polysomic or that the zygote was diploid but asynaptic and univalents divided at metaphase. Thus if one bivalent was formed and disjoined normally, four univalents did not divide and eight did, the result would be twenty-two bodies at anaphase and these could be distributed as in Pl. 1, fig. 5.

Other asci from the same selfing appeared to be abnormal. Pl. 2, fig. 6, which contains fifteen bodies, appears to be a stage earlier than Fig. 5. The fifteen bodies might arise from fourteen univalents, one of which has already divided. Plate 2, fig. 7, contains twelve bodies, possibly two bivalents and ten univalents. In Pl. 2, fig. 8, prophase II appears to have seven bodies at one end and fourteen at the other. This might be normal anaphase II, progressing at the latter end, but it does not appear to be so. In Pl. 2, fig. 9, an ascus from selfing in A.S.F. 10, there appear to be eight bodies at late diakinesis, presumably six bivalents and two univalents.

Altogether fourteen diakinesis—metaphase I configurations and six anaphase I configurations were observed in selfings of A.S.F. strains 10, 11 and 12. Of these only five and two, respectively, appeared to be normal. Although the remainder (see figs. 5–9) were sometimes open to alternative explanations, no similar cases were observed in the cross *pdx-p a* × *Emerson A* and only on two occasions in the cross *pdx A* × *pdx-p a* (see Pl. 1, figs. 2 and 4). It is therefore concluded that meiosis in selfings was very often irregular and that the interpretations of the configurations are similar to those given.

Similar irregularities were observed in a cross between A.S.F. strains 10 and 11. Plate 2, fig. 10, shows an ascospore containing eleven bodies. This might be early anaphase with only four chromosomes disjoined, but it does not appear to be so.

Segregants from selfings of A.S.F. strains were crossed to wild-type strains for mating-type tests and some of these crosses were examined cytologically. Most configurations appeared to be normal but a few showed evidence of univalent formation. Plate 2, fig. 11, is an excellent example of this, there being five unmistakable laggards at telophase I.

DISCUSSION

It seems probable that the behaviour of A.S.F. strains can be explained on the hypothesis that they are disomic for the mating-type chromosome, and probably others, and that the disomic condition is not stable but reverts to haploidy.

Viable ascospores, disomic for the mating type chromosome and heterozygous for mating type might be formed in several ways.

(1) Distribution of univalents during meiosis in a zygote which either has more than two mating-type chromosomes or else has two but is asynpatic or desynaptic. Asynapsis implies that the homologous chromosomes do not pair at zygotene, whereas desynapsis implies that they do, but later fall apart. In desynapsis there is opportunity for chiasma formation and therefore crossing over. Genetical evidence for this has been obtained in asynaptic tomato (Soost, 1951) and in maize (Rhoades, 1947).

(2) Non-disjunction at anaphase of a division reductional for mating type (either anaphase I or II) in a diploid. One would expect this to cause abortion of other spores in the ascus.

(3) Non-disjunction in an interchange heterozygote.

Since the second and third hypotheses would require abortion of some spores in an ascus containing an A.S.F. strain, they are rendered unlikely by such asci as numbers 2 and 4 in Table 4. Recombination between mating-type and growth-rate in each of asci numbers 1, 2 and 4 also indicates that, if the A.S.F. strain is caused by distribution of univalents, then the univalents are formed after crossing-over has taken place. Either the zygote is desynaptic or a univalent has occurred with an homologous bivalent, or a false univalent has been derived from a trivalent.

Assuming that an ascospore giving rise to a self-fertile strains is disomic for the mating-type chromosomes and heterozygous for mating type, i.e. *Aa*, the self-fertile reaction presumably follows the formation of haploid nuclei by rare chromosome loss and selection for this haploid. The mating would then be either *Aa* × *A*, *Aa* × *a* or *A* × *a*. If the mating is between haploid cells, i.e. *A* × *a*, unless the zygote is desynaptic, there is no reason to expect either A.S.F. strains in the progeny or abnormal segregations such as in the ascus containing A.S.F. 9 or those shown in Table 4. Therefore, it seems probable that the mating-type reaction is between a disomic and monosomic or else that it is between two monosomics; and the resulting zygote is desynaptic.

There are three objections to the hypothesis that the mating-type reaction is between a disomic and monosomic. If the mating-type reaction is *Aa* × *A* (or *Aa* × *a*) there is no obvious way to account for a high frequency of second division segregation for mating type. Secondly, A.S.F. strains should be very frequent in the progeny. Thirdly, if the hypothesis that pseudo-wild type strains originate as disomics is correct then it is also correct that disomics for five of the linkage groups, including the one containing the mating type (Pittenger, 1954), do not function in fertilization; this hypothesis requires that they should. This leaves the hypothesis that the mating reaction in an A.S.F. strain is *A* × *a* and the resulting zygote is desynaptic. The cytological observations would agree with this hypothesis because it allows a maximum of fourteen bodies at each pole at anaphase I. This has been observed by Catcheside (1939) in an asynaptic Oenothera which also has a diploid complement of fourteen chromosomes. A high proportion of second-division segregation for mating type means that most of these asci must arise in the following manner. Both univalents divide at the first division of meiosis and are included in telophase nuclei. They do not divide at the second division of meiosis but are distributed so that each telophase II nucleus has one mating-type chromosome. If daughter univalents should divide at the second division of meiosis then they would have replicated once more than, and out of step with, other strands and it is not unreasonable to suppose that they might not replicate at the third division in the ascus. This would result in dissimilar members of a spore pair. For example, the ascus which contained A.S.F. 9 might have arisen as follows. Both univalents divided at anaphase I. One of the *a* daughter univalents was lost; the other was not but did not divide at anaphase II. Both *A* daughter univalents were included in telophase I nuclei and divided again at anaphase II and anaphase III. Thus all spores contained *A* and spore 1 also contained *a*. Spore number 2 probably also contained *a* but self-fertility was not detected.

This hypothesis would adequately account for all observed properties of A.S.F. strains. Simultaneously disomy for more than one chromosome would be expected in some ascospores; this has apparently been observed for the *pdx-p* and mating-type chromosomes in A.S.F. 1, 2, 5 and 6.

Vegetative recombination between the mating-type locus and the unlinked *pdx-p* locus in such an A.S.F. strain should be easily observable in conidia or hyphal tips. A demonstration of this would provide good evidence to support the hypothesis, but it has not been attempted. It is suggested that a similar explanation will account for the formation of pseudo-wild type strains, i.e. that the cross *pdx* × *pdx-p* is partially desynaptic giving rise to occasional disomics. Some cytological evidence for univalent formation in this cross has been presented. That segregants from this cross formed one parent of the crosses from which A.S.F. strains were derived is probably significant but not the whole story. The other parent was either Emerson *A* or Emerson *a*. The author has observed A.S.F. strains in the progeny of the following crosses:

33757 *a* (*leucineless*) × Emerson *A*,
44206 *A* (*adenineless*) × Beadle *a*.

Mr P. Fitzgerald of this department has observed them in the cross Emerson *A* × Emerson *a*, his stocks having been obtained from the author. Moreover, since pseudo-wild type formation is so widespread (Pittenger, 1954), it seems probable that, if desynapsis is the cause, it is common in laboratory stocks of Neurospora.

There are only two reports in the literature of self-fertility in *Neurospora crassa*. Lindegren (1934) attributed it to heterocaryotic ascospore formation. Pateman (1955) observed it but did not make a further report. Probably self-fertility has been observed frequently but attributed to contamination. It is unlikely to be of great use genetically because A.S.F. strains retain their self-fertility for such a short period and are very sterile. However, it should be possible to derive strains of the opposite mating-type which are isogenic (at least temporarily) in all but the mating type chromosome.

Asynapsis has been reported in a wide variety of plants but there is usually no evidence that it is not desynapsis. Asynapsis is frequently inherited as a single gene effect. There is no apparent reason why inherited asynapsis or desynapsis should not occur in Neurospora. Possibly the extreme sterility observed in A.S.F. strains is caused by homozygosity for a gene or genes controlling the desynapsis while in other crosses, such as those giving rise to pseudo-wild type strains, the effect is only partial.

I should like to express my thanks to Professor D. G. Catcheside, F.R.S., and Dr J. M. Mayo for their advice and guidance during the course of this work.

REFERENCES

Barratt, R. W., Newmeyer, D., Perkins, D. D. & Garnjobst, L. (1954). Map construction in *Neurospora crassa*. *Advanc. Genet.* **6**, 1.

Beadle, G. W. & Tatum, E. L. (1945). Neurospora II. Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* **32**, 678.

Catcheside, D. G. (1939). An asynaptic Oenothera. *New Phytol.* **38**, 323.

Lindegren, C. C. (1934). The genetics of Neurospora. VI. Bisexual and akaryotic ascospores from *Neurospora crassa*. *Genetica*, **16**, 315.

Mitchell, M. B. (1955). Aberrant recombination of pyridoxine mutants of *Neurospora crassa*. *Proc. nat. Acad. Sci., Wash.* **41**, 215.

Mitchell, M. B., Pittenger, T. H. & Mitchell, H. K. (1952). Pseudo-wild types in *Neurospora crassa*. *Proc. nat. Acad. Sci., Wash.* **38**, 569.

Newcomer, E. H. (1953). A new cytological and histological fixing fluid. *Science*, **118**, 161.

Pateman, J. A. (1955). Polygenic inheritance in Neurospora. *Nature, Lond.* **176**, 1274.

Pittenger, T. A. (1953). Pseudo-wild type strains in Neurospora. *Genetics*, **38**, 683.

Pittenger, T. H. (1954). The general incidence of pseudo-wild types in *Neurospora crassa*. *Genetics*, **39**, 326.

Rhoades, M. M. (1947). Crossover chromosomes in unreduced gametes of asynaptic maize. *Genetics*, **32**, 101 (Abstract).

Ryan, F. J., Beadle, G. W. & Tatum, E. L. (1943). The tube method of measuring growth rate in Neurospora. *Amer. J. Bot.* **30**, 784.

Singleton, J. R. (1953). The chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. *Amer. J. Bot.* **40**, 124.

Soost, R. K. (1951). Comparative cytology and genetics of asynaptic mutants in *Lycopersicon esculentem*. *Genetics*, **36**, 410.

Strauss, B. S. (1951). Studies on vitamin B6 requiring pH sensitive mutants of *Neurospora crassa*. *Arch. Biochem.* **30**, 292.

Westergaard, M. & Mitchell, H. K. (1947). A synthetic medium favouring sexual reproduction in Neurospora. *Amer. J. Bot.* **34**, 573.

EXPLANATION OF PLATES

Plate 1

Figs. 1, 1*a*. Normal metaphase I in an ascus from the cross *pdx A* × *pdx-p a*. Magnification, ×1200.

Figs. 2, 2*a*. Metaphase I in cross *pdx A* × *pdx-p a* with eight bodies present. Magnification, ×1200.

Figs. 3, 3*a*. Normal anaphase I in cross *pdx A* × *pdx-p a*. Magnification, ×1200.

Figs. 4, 4*a*. Anaphase I in cross *pdx A* × *pdx-p a* showing laggards. Magnification ×1200.

Figs. 5, 5*a*, 5*b*. Early anaphase I in selfing of A.S.F. 11. Twenty-two bodies present. Magnification, ×1200. Fig. 5*a* was taken in a lower focal plane than Fig. 5.

Plate 2

Figs. 6, 6*a*. Metaphase I (approximately) in selfing of A.S.F. 11. Fifteen bodies present. Magnification, ×1200.

Figs. 7, 7*a*. Metaphase I (approximately) in selfing of A.S.F. 11. Twelve bodies present. Magnification, ×1200.

Figs. 8, 8a. Prophase II (approximately) in selfing of A.S.F. 11. Fourteen bodies in one group, seven in the other. Magnification, × 900.

Figs. 9, 9a. Metaphase I in selfing of A.S.F. 10. Eight bodies present. Magnification, × 1200.

Figs. 10, 10a. Ascospore from cross of ASF 10 × A.S.F. 11. Probably metaphase with eleven bodies present. Magnification, × 1200.

Fig. 11, 11a. Telophase I in cross of a segregant from an A.S.F. strain to a wild type strain Magnification, × 1200.

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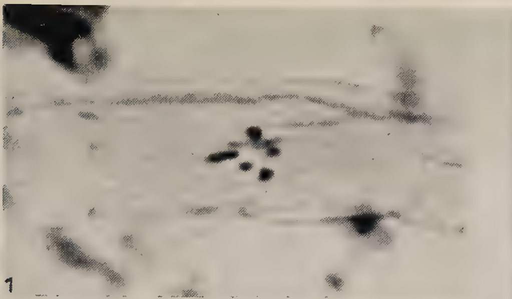
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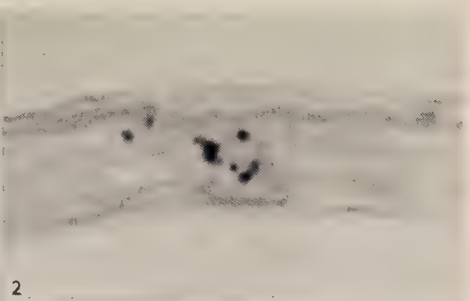
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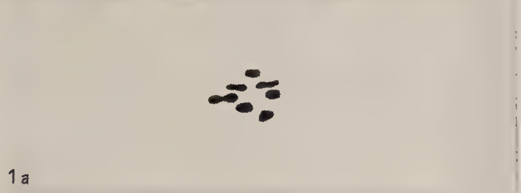
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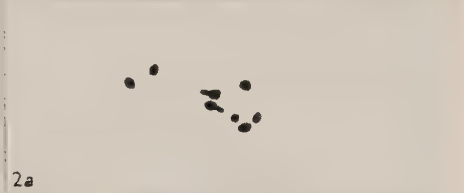
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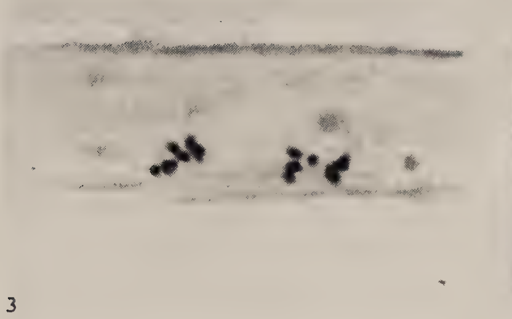
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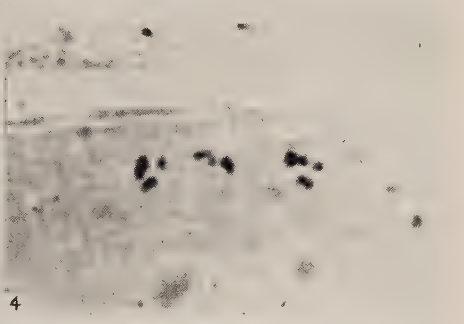
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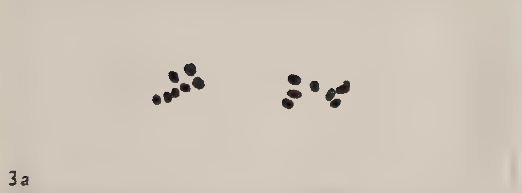
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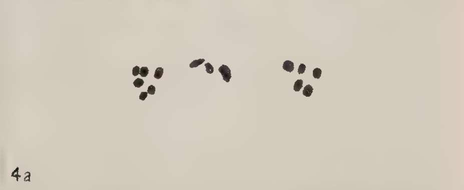
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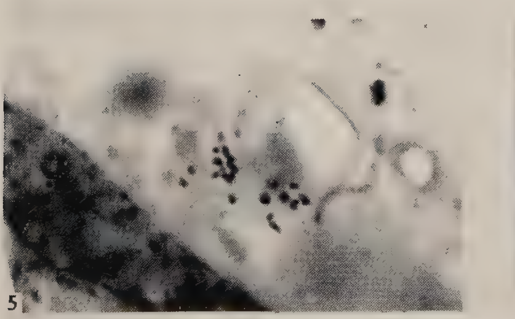
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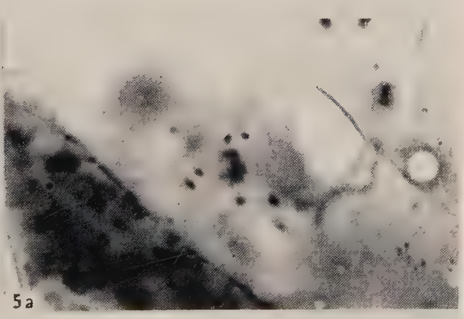
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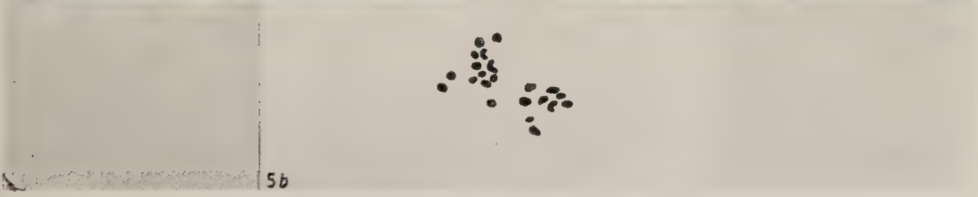
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P. G. Martin—Self-fertility in neurospora. Plate 1

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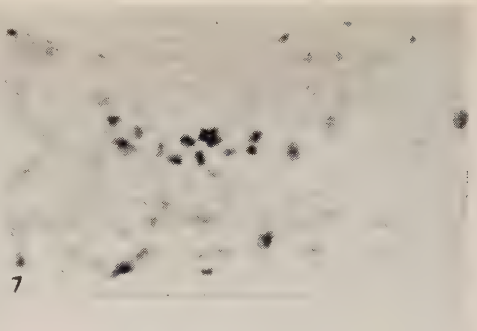
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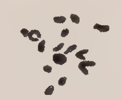
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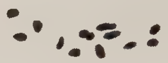
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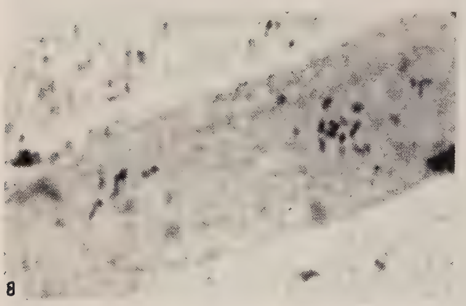
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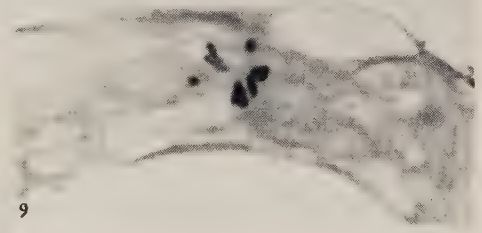
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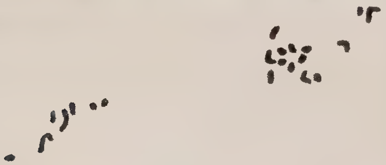
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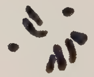
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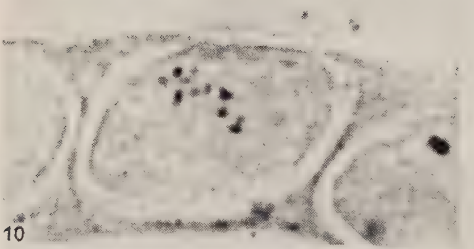
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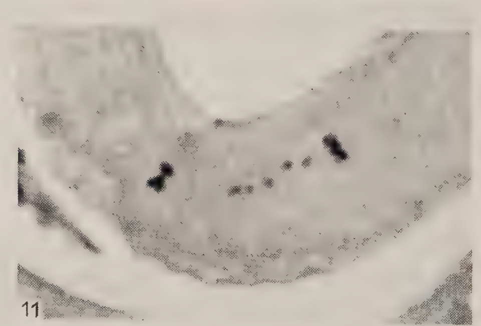
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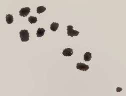
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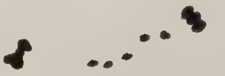
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P. G. Martin—Self-fertility in neurospora. Plate 2

JINKS, J. L. (1959). *J. gen. Microbiol.* **20**, 223–236

Selection for Adaptability to New Environments in *Aspergillus glaucus*

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SUMMARY: Colonies of *Aspergillus glaucus* arising from single asexual spores or hyphal tips of the same or different homokaryon clones often vary in their adaptability to new environments. This shows itself as differences in the percentage survival and mean lag period of inocula taken from colonies growing on a normal medium when transferred to one containing a poison such as mercuric chloride, or unfamiliar sugar sources, e.g. arabinose, galactose, lactose and xylose.

The differences in adaptability to these new media between homokaryon clones are characteristically nuclear in origin, while those between colonies of the same clone are characteristically cytoplasmic. The nuclear system might be formally regarded as epistatic to the cytoplasmic system involved in these adaptive changes.

Selection for the cytoplasmic differences can produce marked changes in the adaptability of the asexual spores of single colonies, even though the selection technique is such that colonies in the direct line of selected descent are at no time exposed to the new media.

The changes in adaptability to mercuric chloride are independent of those for adaptability to the new sugars and only the latter shows any correlation with changes in rate of growth. There is therefore evidence of two or possibly three cytoplasmic systems with much the same properties of variation but independent in action and transmission.

Adaptations to new environments can involve both nuclear and cytoplasmic changes. In *Aspergillus glaucus* the adaptive changes directly responsible for growth in the presence of poisons and unfamiliar sugar sources are confined to the cytoplasm (Briault, 1956). On the other hand, genotypes differ in their ability to adapt to these new media. In the present investigations, therefore, we shall examine the interrelations of nucleus and cytoplasm in determining the ability of an inoculum to adapt to a new environment. At the same time comparisons will be made between the properties of the cytoplasmic system involved in the adaptive changes and of that responsible for the naturally-occurring variation in the morphological characters, rate of growth, perithecial density, pigmentation, etc. With these two aims in view we shall extend the approach developed by Jinks (1954, 1956–58) to investigate the latter system to see how far the explanations and methods of investigation are applicable to the adaptive changes and to establish how far the cytoplasmic systems involved are common to both.

METHOD

Material. The material consists of the wild isolates of *Aspergillus glaucus* described in earlier papers and various derivatives obtained by introducing major gene markers. The latter, which primarily affect spore colour or colonial morphology, have no other significance than that they facilitate the genetic analyses which are carried out from time to time on these isolates to determine the nuclear or cytoplasmic origin of any change in phenotype.

Medium. The basal medium employed throughout was solid Czapek + 5 % sodium chloride. The mercuric chloride was added to this in dosages between $\frac{1}{2}$ and 20 p.p.m. (μg. mg./ml.), while the alternative sugar sources galactose, xylose, arabinose and lactose were each substituted for sucrose at the rate of 2 % by (w/v). The mercuric chloride solution, which was freshly made on each occasion it was used, was sterilized independently of the basal medium and only added to the latter just before pouring into the Petri dishes.

Techniques. The majority of propagations and tests were made on samples of single asexual spores which were prepared, counted and plated as described by Jinks (1952) and Pontecorvo (1953).

The percentage survivals of random samples of asexual spores on the new media are given relative to those of a duplicate sample on the basal medium. Similarly, the mean lag periods, which were obtained by averaging the number of days required for the individual asexual spores to produce a microscopically recognizable colony on the new media, are corrected for the time taken for spores of a duplicate sample to produce similar colonies on the basal medium.

Occasionally hyphal propagations were used and unless otherwise stated the inocula contained approximately fifty hyphal growing-tips taken from the periphery of a colony. In one experiment, the results of which are summarized in Table 4, inocula containing different numbers of hyphal growing-tips were used. The latter were counted under the microscope for those inocula which contained 4, 16 and 32 intact growing-tips, including in the counts only those tips which had not lost their contents as a result of cutting. The larger inocula, however, were estimated as that sized arc of the periphery of a colony which on the average was found to contain 50, 100, 200, etc., intact growing-tips on counting a duplicate sample.

Phenotypic differences of nuclear and cytoplasmic origin were separated by the heterokaryon test, the various aspects of which have been described in a number of papers (Jinks, 1954, 1956, 1958: Arlett, 1957; Gibson & Griffin 1958).

RESULTS

Differences between homokaryon clones

Homokaryon clones differ in their adaptability to the new environments on both criteria used, viz. the percentage survival of transfers to the new media and the duration of the initial lag period. Examples, using random samples of asexual spores, produced on the normal medium as inocula, are given in Tables 1 and 2. In these tables the percentage survival at various concentrations of mercuric chloride and the mean lag of the survivors are given for three homokaryon clones.

Similar, but on the whole less marked, differences between homokaryon clones were obtained on the new sugars where the survival rates were high and the lag period of short duration.

These differences in adaptability between homokaryon clones, which of course are found with hyphal as well as spore inocula, can be analysed in such a way as to determine their nuclear or cytoplasmic origin (Jinks, 1954, 1956, 1957, 1958).

Table 1. *The percentage of asexual spores in random samples from three homokaryon clones which successfully adapted, i.e. formed colonies on 2, 3·5 and 5 p.p.m. of mercuric chloride*

Homokaryon:	2.1	2.w.	1.s
Conc. of mercuric chloride (p.p.m.)	Percentage of random sample		
2	67·2	69·6	36·5
3·5	51·4	62·5	19·5
5	6·8	44·7	10·0

Table 2. *Mean lag period in days of those asexual spores from three homokaryon clones which were capable of growth on 2, 3·5 and 5 p.p.m. of mercuric chloride*

Homokaryon:	2.1	2.w	1.s
Conc. of mercuric chloride (p.p.m.)	Mean lag in days		
2	3·32	1·45	2·87
3·5	3·97	2·71	4·73
5	7·30	5·91	7·32

The technique may be illustrated by reference to clones 2.w and 1.s (Tables 1 and 2). These two homokaryons were readily distinguishable morphologically both from one another and from the heterokaryon which was easily formed between them. Since the heterokaryon produced a high proportion of homokaryotic spores the two homokaryons could be re-isolated from their heterokaryotic association following a period of co-existence of the two kinds of nuclei in the same cytoplasm. The adaptability of the homokaryons can, therefore, be compared before and after the heterokaryotic association of their nuclei. Table 3 shows the results of one such comparison of the adaptability of homokaryon 1.s before and after heterokaryotic association with homokaryon 2.w. The character is the percentage of asexual spores in random samples which successfully adapt to three different levels of mercuric chloride.

Table 3. *The percentage of asexual spores in random samples from homokaryon 1.s which successfully adapted to 2, 3·5 and 5 p.p.m. of mercuric chloride before and after heterokaryotic association with homokaryon 2.w.*

	Percentage of random sample	
Conc. of mercuric chloride (p.p.m.)	Before heterokaryon	After heterokaryon
2	35·8	37·2
3·5	19·0	20·0
5	8·3	11·6

The results summarized in Table 3 were analysed by a contingency χ^2 and a probit analysis. These analyses agreed in showing no change as a result of the heterokaryotic association. Also in agreement was an analysis of variance of the angular transformed percentage survivals which is given below.

Item	Degrees of freedom	Mean square
(1) Conc. of poison	2	705·2
(2) 'Before' vs. 'After'	1	10·4
1 × 2	2	5·2
Duplicates	24	18·2

The other component of the heterokaryon, homokaryon 2.w, also showed no change in adaptability following its association with homokaryon 1.s and indeed this was the general finding from such comparisons. It would seem, therefore, that the differences in adaptability between these homokaryon clones are characteristically nuclear in origin.

Differences within homokaryon clones

The results in Table 1 show that there was considerable heterogeneity in the ability of asexual spores of uniform age, and produced by single homokaryon colonies to adapt, i.e. survive and grow, at different concentrations of mercuric chloride. There is also heterogeneity, although less marked, in the ability of similar samples of asexual spores to adapt to new sugars (Briault, 1956).

These results, however, by no means convey the full extent of this heterogeneity among single asexual spores, for even among those spores which could adapt to the new conditions there occurred considerable variation in the duration of the initial lag period. There is, of course, some variation in the onset of germination among single asexual spores even on the normal medium, but this is regularly increased up to fourfold on the adaptive media.

Direct evidence of heterogeneity between the vegetative hyphae of single homokaryon colonies was more difficult to obtain, mainly because propagations by single vegetative hyphae resulted in mechanical damage to the colony and the transferred cells. As a consequence it was not always easy to separate failure to adapt, which led to death, from death resulting from mechanical damage.

One approach has been to investigate the effect of the number of hyphal growing tips in an inoculum on their survival and on duration and variation in the lag period on mercuric chloride. As a control, to determine effects due to the experimental procedure, duplicate inocula were placed on the normal medium and their performance used to correct the results obtained on the poison.

In general, inocula containing fifty or more intact growing-tips always successfully adapted to quantities of poison as high as 5 p.p.m. after a uniform lag period of 3–4 days, the details varying between different homokaryon clones. With smaller inocula there were frequent failures to adapt as well as variation in the duration of the lag period of those which did adapt. An example of the type of results obtained is given in Table 4. These relate to transfers of homokaryon 2.10 from the normal medium to 5 p.p.m. of mercuric chloride.

Table 4. *The effect of inoculum size on mean lag periods*

Number of intact growing tips in inoculum	Normal medium	5 p.p.m	Lag due to poison
	Mean lag in days		
4	5·6	17·0	11·4
16	2·9	12·5	9·6
32	2·1	9·6	7·5
50–200	2·0	5·3	3·3

Not only had the mean lag period, after correction, more than trebled from the largest to the smallest inoculum, but the variation in the lag periods of replicate inocula increased fivefold over the same range. More important, however, while all inocula with more than 32 intact tips adapted, 6 out of 13 and 6 out of 15 of the 4 and 16 tip inocula failed to grow. Pooling the results from these two sizes of inoculum, 12 grew and 2 failed on the normal medium while 4 grew and 10 failed on the poison. On both media deaths will have occurred from mechanical damage and other upsets resulting from the experimental procedure, but the significant difference ($P = 0{\cdot}01 - 0{\cdot}001$) between the frequency of deaths on the two media must be specifically related to the inability of some undamaged cells to adapt to the presence of the poison. Such evidence can only imply differences between the vegetative hyphae of single homokaryon colonies in their ability to adapt to the new medium.

An analysis of the origin of this heterogeneity between both single asexual spores and vegetative hyphae from individual homokaryon colonies is described in later sections where the former variation is used as the basis of selection experiments.

Selection for adaptability to the presence of mercuric chloride

It was shown (Jinks, 1957) that there was variation in morphological characters among the asexual progeny of single homokaryon colonies, and that selection for the extreme variants could give rise to large differences in phenotype depending solely on differences in cytoplasmic content. Similar selection has been used to investigate the variation in adaptability between colonies derived from the asexual spores of individual homokaryon colonies.

Each cycle of selection was initiated by raising large numbers of sister colonies on the normal medium each from a single asexual spore produced by a single parent homokaryon on the same medium. Five of these sister colonies were chosen at random and samples of their asexual spores simultaneously tested for their ability to adapt to two or more levels of mercuric chloride. In the first cycle the two colonies, whose asexual spores gave the highest and lowest percentage survival, respectively, were retained for the second cycle. In the second, and subsequent cycles, the asexual spores of the two sets of five colonies were simultaneously tested and only one from each set retained, namely, the highest of the five from the high line and the lowest of the five from the low line. An unselected control line was maintained in the same way, except that in each cycle one colony was chosen at random out of the five tested instead of on the basis of the performance of its asexual spores on the poison. No colony in the direct line of selected descent was exposed to the poison: their change cannot, therefore, be referred to a direct action of the poison. Selection was in fact practised for adaptability rather than adaptation.

A typical example is the selection for high- and low-percentage adaptation to mercuric chloride of the asexual spores of homokaryon 1.5. Selection was here carried out for four cycles at two or three concentrations of mercuric chloride, chosen so that one lay on either side of the 50 % survival point. To achieve this it was necessary to adjust the concentrations of mercuric chloride used in successive cycles, basing the adjustments on performance in the previous cycle. All the concentrations used, however, fell within the range 2–5 p.p.m. The final comparison of the selections was made at 5 different concentrations of mercuric chloride, ranging from 0 to 5 p.p.m.

The estimated LD 50 values for the high and low selections and the control line are given in Table 5.

Table 5. *The LD 50 values in p.p.m. of mercuric chloride for the high and low selections and the control line derived from homokaryon* 1·5

	Parent:	1	2	3	4
Cycles of selection		LD 50 value, in p.p.m. mercuric chloride			
High line	—	3·17	4·43	4·34	4·62
Control line	3·62	3·13	3·55	4·03	3·70
Low line	—	3·28	3·65	3·81	3·04

The results in Table 5 show that marked progress was made after only four cycles of selection; a highly significant difference of 1·52 ± 0·18 was achieved between the high and low lines. Moreover, taking the control rather than the parental value as a base line, progress was made in both directions.

Two attempts were made to estimate the heritability of this character during the course of the selections. The first was made between the first and second cycles when the colonies chosen to continue the selections were supplemented by others chosen at random. In all, eleven colonies from the first cycle were used as parents, three from each of the selections and five from the control. The correlation coefficient between the performance of parent colonies in the first cycle and the mean performance of their daughter colonies in the second was not significant ($r = 0·33$). When, however, we average the performance within each of the selection lines and within the control line the parent/daughter correlation is 0·93. Repeats on a smaller scale for the second and third and third and fourth cycles gave correlations of 0·98 and 0·66, respectively, the former being highly significant.

The second estimate was a comparison between the difference in performance of the parents chosen to continue the high and low selections (the selective differential) and the observed difference in performance of random samples of the asexual spores of five of their daughter colonies in the next cycle. Over the four cycles of selection the regression of observed difference on the selective differential was 0·96 ± 0·36, the expected value being 1·00 if all the differences between parent colonies were heritable.

The mean lag period of those transfers which successfully adapted was scored throughout the selections and the results for the highest concentration of poison used in each cycle are given in Table 6. Some of the variation between cycles for this character was undoubtedly due to the deliberate variations in the concentrations of poison used (as described earlier), but within each cycle the comparison between lines was independent of this variation.

Table 6. *The mean lag period in days of the asexual spores of the high, low and control lines of homokaryon* 1·5 *which successfully adapted to the highest concentrations of poison used in each cycle*

Cycles of selection:	1	2	3	4
	Mean lag in days			
High line	4·86	2·99	3·84	2·78
Control line	4·55	2·55	3·96	4·87
Low line	4·25	5·42	6·21	6·31

Clearly, the two estimates of the adaptabilities of the selection lines, namely, their LD 50 values (Table 5) and their mean lag periods (Table 6) are highly negatively correlated and hence provide much the same picture of the progress of selection. The negative correlation is general throughout the data, being present in every individual sample of five sister colonies scored during the selections. But, as might be expected with such small samples, the negative correlations are significant only in four of them.

The two homokaryons re-extracted in the form of asexual spores of the heterokaryons between colonies of the high and low selections no longer showed the difference in adaptability to mercuric chloride. This is shown by the following analysis of variance of the angular transformed percentage adaptations of asexual spores taken from the re-extracted homokaryons when plated on 3 and 5 p.p.m. of the poison.

Item	Degrees of freedom	Mean squares
(1) Differences between homokaryons after re-extraction from heterokaryon	1	34·99
(2) Concentrations of poison	1	4.641·18
(3) Replicate concentrations of poison	2	10·79
1 × 2	1	3·42
1 × 3	2	38·14

None of the highly significant differences in adaptability survived the co-existence in the heterokaryon state. These differences must, therefore, be cytoplasmic in origin.

Further heterokaryons were made between colonies of the high or low selections and unselected colonies of independent clones, e.g. homokaryon 2.6. The LD 50 values of the asexual spores of the homokaryons re-extracted from the heterokaryons in one of these experiments are listed in Table 7. For comparison the LD 50 values of the selection lines before heterokaryon formation are listed in the first column of this Table.

Table 7. *The effect of heterokaryotic association on the LD 50 values of the high and low selections of homokaryon* 1·5 *and a normal colony of homokaryon* 2.6

	LD 50 values (p.p.m. of mercuric chloride) and their standard errors		
	Selection lines of homokaryon 1.5		Normal homokaryon 2.6
Heterokaryon	Before heterokaryon	After heterokaryon	After heterokaryon
High selection + homokaryon 2.6	4·62 ± 0·12	3·92 ± 0·05	4·21 ± 0·05
Low selection + homokaryon 2.6	3·04 ± 0·19	3·67 ± 0·06	4·02 ± 0·06

The difference between the LD 50 values of the selection lines and homokaryon 2.6 after extraction from the heterokaryons must be under nuclear control. The lowering of the LD 50 values of the high selection and the raising of that of the low selection so that both approach the value for the control line (3·63 ± 0·07) must, on the other hand, be attributed to exchanges between the selected and wild-type cytoplasms while in the heterokaryons. A small part (16 %) of the difference in LD 50 between the high and low selections has, however, survived heterokaryotic association with the normal cytoplasm of homokaryon 2.6. Similarly, a difference of the same magnitude and sign has arisen between the two samples of homokaryon 2.6 following their association with the high and low selections in the heterokaryons. These persistent differences between the selection lines and the new differences within homokaryon 2.6 must be related to cytoplasmic differences between the two heterokaryons, which in turn could only have been introduced by their non-common component, namely the selected lines of homokaryon 1.5.

Attempts to select for differences in adaptability to mercuric chloride in homokaryon 2.6 failed where the procedures were identical with those just described for homokaryon 1.5. Modification of the procedures so as to increase the selection pressure might well have produced a response in homokaryon 2.6. But this would have upset one of our aims, namely to compare the two genotypes of homokaryons 1.5 and 2.6 under identical conditions of selection. A number of experiments described later demonstrate conclusively that cytoplasmically differentiated lines can be obtained within homokaryon 2.6, which differ significantly in the ability of their asexual spores to adapt to mercuric chloride.

Selection for adaptability to new sugars

We may recognize two types of difference in adaptability to new sugars between samples of asexual spores taken from sister colonies of a homokaryon clone. Either of these differences, which will be referred to as general and specific, might be utilized as the basis of a selection experiment. General adaptability refers to the mean adaptability of a sample of asexual spores when tested on a number of new sugars. Specific adaptability, on the other hand, refers to an above or below average performance of a particular sample of asexual spores on a particular new sugar. We can illustrate this distinction by reference to tests of the asexual spores of five asexually-derived sister colonies of homokaryon 2.6 on the four sugars, galactose, arabinose, xylose and lactose. The results, given in Table 8, are the numbers of normal colonies, produced by the five samples of asexual spores when equally divided between the four sugars.

Table 8. *The totals of successful adaptations when samples of asexual spores from five sister colonies of homokaryon* 2.6 *on the normal medium were equally divided between media containing four new sugars*

Colony	Galactose	Arabinose	Xylose	Lactose	Total	Expected totals
1	292	272	328	137	1029	902
2	160	204	224	66	654	943
3	142	214	182	47	585	560
4	254	326	341	168	1089	964
5	169	147	183	52	551	539
Totals:	1017	1163	1258	470	3908	

Differences in general adaptability were detected by comparing the observed and expected row totals in Table 8 by a χ^2 for 4 D.F., the expected totals being estimated from the relative number of asexual spores in each of the samples taken from the five colonies.

By using the observed column and row totals we can test for heterogeneity between the performance of these samples on the four sugars, i.e. for differences in specific adaptability, by calculating the interaction χ^2 for 12 D.F.

Analysis of the data in Table 8 gives the following results:

	χ^2	N	P
Specific adaptability	52·57	12	$<0{\cdot}001$
General adaptability	124·05	4	$<0{\cdot}001$

Clearly, we have both specific and general differences in adaptability between the five sister colonies of homokaryon 2.6. A major contribution to the differences in specific adaptability between the five colonies comes from the tests on the two sugars xylose and arabinose, the asexual spores of some colonies (e.g. 1 and 5 in Table 8) adapting more readily to xylose while those of other colonies show no preference or a slight one for arabinose. A selection experiment was, therefore, initiated in homokaryon 2.6 where the high line was for high adaptability to arabinose and low to xylose, and the low line high adaptability to arabinose and low to xylose. General adaptability, measured as the mean adaptability to both sugars, was scored as a secondary character. The actual selective procedures were those described earlier for adaptability to mercuric chloride.

There was a marked, although asymmetrical, response to selection. Thus in the parent clone xylose was already at an advantage as an alternative sugar ($X/A = 1{\cdot}21$) and the high selection only raised this to 1·29. The low selection, however, produced a greater response and over three cycles, the value of X/A fell to 0·84.

General adaptability was correlated with the ratio X/A. In the first cycle of selection this correlation was as high as −0·87, suggesting that only colonies with high general adaptability could adapt to arabinose as well as they did to xylose. This negative correlation persisted throughout, although it reached a value as low as −0·22 in the sample of asexual spores taken from the colonies of the third cycle of selection.

Mean lag period, the other metric for assessing differences in adaptability, was scored throughout the selections. In the parent clone the mean lag on xylose was 4·56 days shorter than that on arabinose. In the high line this advantage in mean lag was raised to 5·36 days while in the low line it was reduced to 3·99 days. Thus this metric also shows a response to selection for differences in adaptability to xylose and arabinose.

Neither significant general nor specific differences in adaptability to the four new sugars were detected in three successive samples of five sister colonies of homokaryon 1.5; hence attempts to select within this clone were abandoned.

Adaptability to mercuric chloride and the new sugars

The differences between the selections for high and low adaptability to mercuric chloride and to the new sugars are solely cytoplasmic in origin. We may now inquire whether the cytoplasmic systems involved are common to both the adaptability to the poison and to the sugars or whether the two are independent. Direct evidence on this point can be obtained by scoring adaptability to both the poison and the sugars on the same samples of asexual spores, the latter being taken either from random samples of sister colonies or from selections for adaptability to one or other of the environmental changes.

Five sister colonies of homokaryon 2.6 were chosen to give a highly significant difference both in general and specific adaptability to the sugars ($P < 0{\cdot}001$ for both). Simultaneous with the tests on the sugars, asexual spores of the same samples were also tested on two levels of mercuric chloride, viz. 3 and 5 p.p.m. Analyses revealed no significant differences in adaptability to the poison. An identical result was obtained by using the mean lag periods on the sugars and poison as the metric. Again, there were highly significant differences between the five colonies on the sugars ($P < 0{\cdot}001$) but not on the poison. These, and similar findings from other experiments, leave little doubt that adaptability to the poison and sugars have independent cytoplasmic origins. This is confirmed in the next section.

The adaptive and morphological systems

It was observed (Jinks, 1957) that variation in rate of growth, perithecial density, pigmentation, etc., was highly correlated and apparently controlled by the same cytoplasmic system. Whether this system is independent of those controlling adaptability to the poison and the sugars was now examined.

One way of making this comparison is to score the rate of growth of the lines selected for differences in adaptability. As an example, five colonies were chosen at random from the high, low and control lines of homokaryon 1.5 after 4 cycles of selection for differences in adaptability to mercuric chloride. The experiment was replicated four times by single asexual spore transfers and each replicate duplicated by mass hyphal transfers; all colonies, amounting to 120, were grown both on the normal medium and on 4 p.p.m. of the poison.

Analyses of the total growth over three successive days showed that significant differences in rate of growth had arisen both within and between the selection and control lines.

		Medium	
Item	Degrees of freedom	Normal mean squares	4 p.p.m. $HgCl_2$ mean squares
Between lines	2	4·13	16·27
Within lines	12	3·83	2·41
Replicates	45	1·22	0·89
Duplicates	60	0·29	0·41

The five colonies chosen to represent each line were also scored for the percentages of their asexual spores which were capable of adapting to 4 p.p.m. of the poison and the correlation between this and their rates of growth was determined (Table 9).

Table 9. *Correlations between percentage adaptation of asexual spores on* 4 *p.p.m. mercuric chloride and the growth rate on the normal medium*+4 *p.p.m. mercuric chloride for the selections and control line of homokaryon* 1.5

	Medium	
Line	Normal	4 p.p.m.
High	+0·48	+0·06
Control	−0·43	−0·71
Low	+0·28	+0·35
Overall	0·00	−0·09

None of the correlations in Table 9 is significant.

A similar result was found by comparing the adaptabilities of lines selected for differences in rate of growth. In some cases these selections affected the ability of their asexual spores to adapt to mercuric chloride. But results such as those given in Table 10, which were obtained from three selections for fast and slow growth in homokaryon 2·6 show that the correlation between rate of growth and the ability to adapt to the poison must be low.

In other cases selections for fast and slow growth left adaptability unaffected. Such an example is provided by further selections of homokaryon 2.6, three slow and two fast, with rates of growth as different as 1·2 and 2·4 mm./day, respectively. Their adaptive properties were tested on both 3 and 5 p.p.m. of the poison. There were no differences in adaptability between selections

($P = 0{\cdot}95$) and a doubtfully significant difference within them ($P = 0{\cdot}05$). As a consequence the correlations with rate of growth were not significant, being 0·24 and −0·40 for the two concentrations of poison.

Table 10. *The percentage adaptation and mean lag of random samples of asexual spores of three independently derived fast and slow selections of homokaryon* 2.6

Selection		Percentage adaptation to 3 p.p.m. $HgCl_2$	Mean lag in days on 3 p.p.m. $HgCl_2$
Fast		20·4	5·73
		19·3	6·04
		14·0	4·89
	Mean	17·9	5·55
Slow		38·5	4·26
		3·8	7·34
		2·7	9·30
	Mean	15·0	6·97

In contrast, adaptability to the new sugars was in general more highly correlated with rate of growth. For example, analysis of the adaptabilities to the four sugars of the fast and slow selections of homokaryon 2.6 showed highly significant general and specific differences ($P < 0{\cdot}001$ for both), the correlation between rates of growth and general adaptability being 0·90 ($P = 0{\cdot}05$–$0{\cdot}02$). The same high correlation also applied to specific adaptability, the best of the four sugars for the fast selection being consistently galactose and for the slow selection, lactose.

The other metric used for adaptability, lag period, gave similar results, the mean lag on the four sugars of the fast and slow selections being 5·65 and 7·35 days, respectively. A comparison of the rates of growth and the mean lag on all sugars gave a high negative correlation of −0·72.

The cytoplasmic systems

Before discussing the contribution of the cytoplasm to variation in adaptability to new media it is worth noting that no nuclear contribution to intra-clonal variation for this character was found during extensive testing. Nevertheless, the same testing techniques have shown (see *Differences between homokaryon clones*) that the differences between the parental clones for this character were, as one might expect, characteristically nuclear in origin. The phenotypic differences produced by the latter may be as large as any which can be achieved by variation in the cytoplasmic systems and they appear to exert considerable influence on the outcome of the selections for differences of cytoplasmic origin; so much so, that the nuclear system might be formally described as epistatic to the cytoplasmic system involved in the adaptive changes.

The cytoplasmic systems involved in the differences in adaptability have the same general properties as those responsible for the variation in rate of growth, perithecial density and pigmentation, which arise during differentiation and ageing (Jinks, 1956). Thus they produce continuous variation in the adaptability of sister colonies derived from single asexual spores or single vegetative cells. From the behaviour under selection it is clear that these differences persist in asexual propagation and can be accumulated to give smooth changes in phenotype. The absence of segregation for quantitative differences in adaptability in the asexual progenies of single colonies both before and during the heterokaryon tests suggests that major cytoplasmic mutations are not involved in the variation, which probably, therefore, depends solely on differences in the equilibria of normal cytoplasmic constituents (Arlett, 1957; Jinks, 1958).

An important feature of the phenotypic variation in morphology that arose from cytoplasmic changes during differentiation and ageing was the high correlation between all the characters followed: all changed when any one was selected. Whether, however, this was true pleiotropy or sprang from mechanical association of the determinants in transmission could not be decided.

In the present work lines selected for differences in adaptability to new media showed significant differences for the other characters followed. Nevertheless, the correlations between the selected and unselected characters are in general quite low. This is particularly true of the homokaryon 1.5 lines selected for differences in adaptability to mercuric chloride, where the overall correlations with rate of growth were virtually zero (Table 9). The same is true of the reverse selections for homokaryon 2.6, where lines showing the maximum differences in rate of growth, obtainable by selection, showed no consistent differences in adaptability to mercuric chloride.

The correlations between rate of growth and adaptability to the new sugars are quite high ($r = 0{\cdot}8$–$0{\cdot}9$) and there is every indication that in general the more vigorously a colony grew on the normal medium the greater the adaptability of its asexual spores to the new sugars. In view of this difference in relationship between rate of growth and adaptability to the new sugars, on the one hand, and rate of growth and adaptability to mercuric chloride on the other, it seems clear that adaptability to the new sugars must be independent of adaptability to mercuric chloride.

While our primary concern was with cytoplasmic inheritance, of which adaptability to new media is just one more easily demonstrable example, our results, nevertheless, have a bearing on the nuclear basis of adaptation.

The importance of the nuclear component, which we have suggested is epistatic to all others, has been illustrated by clonal differences for the ability of asexual spores and vegetative cells to adapt to new media. But we have also shown that possession of the right nuclear genes by a spore or cell is not in itself sufficient to ensure successful adaptation. A cytoplasmic component is also required and spores and vegetative cells may be deficient or unbalanced for this component.

Adaptation is thus basically the selection of those cells, spores or colonies which possess a nuclear-cytoplasmic combination capable of producing the necessary re-adjustments required for successful growth in the new environment. And although in the wild state this selection would be automatically applied by the new environment itself, conscious selection in the old environment, as described in this paper, can significantly affect the chances of survival on exposure to a new one.

REFERENCES

Arlett, C. F. (1957). Induction of cytoplasmic mutations in *Aspergillus nidulans. Nature, Lond.* **179**, 1250.

Briault, P. G. (1956). Cytoplasmic changes associated with adaptation and differentiation. *Nature, Lond.* **178**, 1223.

Gibson, A. & Griffin, D. M. (1958). Cytoplasmic variation in *Nectria stenospora. Nature, Lond.* **181**, 1222.

Jinks, J. L. (1952). Heterokaryosis; a system of adaptation in wild fungi. *Proc. Roy. Soc.* B, **140**, 83.

Jinks, J. L. (1954). Somatic selection in fungi. *Nature, Lond.* **174**, 409.

Jinks, J. L. (1956). Naturally occurring cytoplasmic differences in fungi. *C.R. Lab. Carlsberg,* (*Ser. Physiol.*) **26**, 183.

Jinks, J. L. (1957). Selection for cytoplasmic differences. *Proc. Roy. Soc.* B, **146**, 527.

Jinks, J. L. (1958). Cytoplasmic differentiation in fungi. *Proc. Roy. Soc.* B, **148**, 314.

Pontecorvo, G. (1953). The genetics of *Aspergillus nidulans. Advanc. Genet.* **5**, 141.

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Strauss, B. S. & Minagawa, T. (1959). *J. gen. Microbiol.* **20**, 237–245

The Formation of Methionine by a Methionine-Requiring Mutant of *Neurospora crassa*

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SUMMARY: A methionine-requiring mutant of *Neurospora crassa* incorporated $^{35}SO_4$ into methionine. This incorporation represents net synthesis of methionine by the mutant. It is quite likely that methionine synthesis proceeds by the normal pathway in the mutant. Not all of the normal proteins can be synthesized when methionine supplies are limiting, accounting for the inability of the mutant to begin growth without a long lag even though methionine can be synthesized.

In the course of our investigations on the lethal effect of incorporated ^{35}S in Neurospora (Strauss, 1958*b*) we decided to study the effect of sulphur distribution on the radiation sensitivity of conidia. It seemed likely that we could compare the relative 'importance' of the incorporation of ^{35}S into cysteine or into methionine by comparing the lethality of $^{35}SO_4$ incorporated into a wild type and into a methionine-requiring mutant. We supposed that the wild type would incorporate sulphur into protein cysteine and methionine but that the mutant would incorporate sulphur only into cysteine. The growth characteristics of the mutant indicated that it should not be able to synthesize methionine. When the experiment was done we found that there was very little difference in the distribution of labelled sulphur in the conidia of the mutant compared to the wild type grown with $^{35}SO_4$ and non-labelled methionine. Protein hydrolysates from both these strains appeared to have ^{35}S incorporated into methionine. Since this behaviour was unexpected we decided to investigate this methionine-requiring strain in more detail. Although 'leaky' mutants have been described before (Bonner, 1951; Bonner, Yanofsky & Partridge, 1952) we hoped to be able to account for the ability of a mutant to synthesize the substance it requires for growth although unable to grow in the absence of added substance.

METHODS

The methionine-requiring mutant used in these studies was received from Dr A. Srb bearing the isolation number 584. Dr Srb informed us that mutant 584 was obtained by the method of Woodward, De Zeeuw & Srb (1954) following ultraviolet irradiation of wild-type conidia of *Neurospora crassa*. Crosses of the mutant to wild-type indicated no significant deviation from the expected ratio in the progeny from a single factor cross. Of the limited number of selected ascospores which germinated and were tested there were ninety-one wild-type and eighty-seven mutant cultures obtained. There were no obvious differences between the mutant progeny when incubated in minimal medium or with methionine. Strain 584 shows no growth after 3 days in minimal medium at 30° (Beadle & Tatum, 1945). Good growth was obtained on the addition of L- or DL-methionine to minimal medium or on the addition of either methionine sulphoxide or homocysteine. No growth was obtained when either cystathionine, homoserine, cysteine, cystine, taurine, cysteic acid, thiosulphate or sulphite was added to minimal medium. The mutant acted as though it had a genetic block in the conversion of cystathionine to homocysteine. A typical growth-response curve was obtained when the dry weight of mycelium produced after 3 days growth at 30° is determined as a function of methionine concentration. The final dry weight attained was independent of the amount of methionine added; once growth had begun it continued until a maximum weight was reached (Fig. 1).

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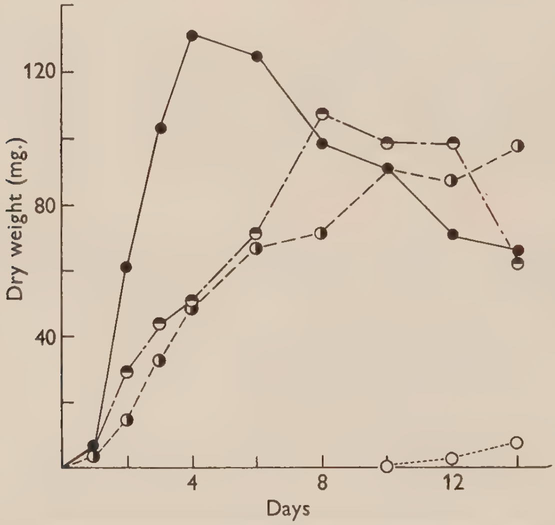

Fig. 1. Growth of the methionine-requiring Neurospora mutant with different amounts of L-methionine. Growth was determined as mg. dry weight of mycelium produced after incubation for the indicated period of time in standing culture at 30° in 125 ml. Erlenmeyer flasks containing 20 ml. minimal medium + the following additions: ○—○ = no addition; ●—● = +10 mg. L-methionine; ◓—◓ = + 1·0 mg. L-methionine; ◑—◑ = +0·5 mg. L-methionine.

Measurement and location of ^{35}S *and* S-*containing compounds.* In the experiments which follow the methionine-requiring mutant and the wild-type were grown, or incubated, in a medium modified to permit control of the amount of sulphur added. $MgCl_2$ was substituted for the corresponding sulphate in minimal medium and a trace element supplement was used which contained no sulphur (Strauss, Vaharu & Frickey, 1956). Carrier-free $^{35}SO_4$ was obtained from Oak Ridge and diluted to an appropriate specific activity. Conidia were obtained as described previously (Strauss, 1958*a*). Our methods for handling Neurospora were those in standard use. Mycelia or conidia were fractionated for the identification and determination of sulphur compounds by a slight modification of the methods described by Roberts *et al.* (1955). The protein amino acids were obtained by extracting the mould with aqueous ethanol, then with warm ethanol + ether (3:1) and with warm (70°) trichloroacetic acid. The residue was washed, dried and then hydrolysed with 6N-HCl by autoclaving at 15 lb./sq.in. for 2 hr. in a glass-stoppered tube. The hydrolysate was evaporated to dryness several times *in vacuo* over phosphorus pentoxide and sodium hydroxide in order to remove excess HCl. The resulting amino acid mixture was then made to volume and chromatographed in a descending system in a phenol + water mixture. Radio-active areas on the chromatograms were detected by scanning with a 'Forro' chromatogram scanner (Forro Radiochemical Co., Chicago) connected to an Esterline-Angus recorder (Esterline Angus Co., Indianapolis) through a Nuclear ratemeter (Nuclear, Chicago). The radio-active areas were occasionally eluted for further analysis. Radio-active free amino acids were extracted by treating the mould with 70% (v/v) ethanol in water.

Recognition of the two sulphur amino acids found in protein is relatively simple, since the amino acids of the cysteine family have low R_f values when chromatographed in a phenol + water system, while the methionine family has the relatively high R_f value characteristic of the more aliphatic compounds (Block, Durrum & Zweig, 1955). The sulphur amino acids may exist in a variety of oxidized forms; methionine, for example, may be identified as the sulphoxide or the sulphone. The exact proportion of each form obtained seems to depend on the conditions of hydrolysis. Many workers prefer to complete the oxidation before chromatography but we chromatographed our material directly and attempted to identify the particular oxidation products by chromatography in a second solvent system which separates the oxidation forms. Since we were unable to find a solvent system which satisfactorily separated the cysteine family from cystathionine a paper electrophoresis method was used. Electrophoresis at 14 v./cm. on Whatman 3MM paper (hanging freely from supports) for 4–5 hr., using 0·25M-acetic acid (pH 2·8) separated cysteine and cystathionine. The cystathionine moved an average of 3·6 cm./hr., the cysteine 3·0 cm./hr., under these conditions.

RESULTS

Mutant conidia, obtained from cultures grown with 2·5 μmole $^{35}SO_4$ plus 4 μmoles of non-labelled methionine in a modified liquid Westergaard medium (Strauss *et al.* 1956), contained labelled methionine. The scanning pattern of the mutant protein hydrolysate was only slightly different from the wild-type pattern. In several experiments we inoculated the conidia, produced during growth of strain 584 with $^{35}SO_4$, into minimal medium. No growth resulted until methionine was added, indicating that the conidia were still mutant.

Wild-type (74A) incorporated more $^{35}SO_4$ into the methionine of its protein than into the cysteine. This pattern applied also to uniformly-labelled conidia as well as to non-labelled conidia incubated for 2 hr. in sulphur-limiting medium. There was a difference in the distribution of incorporated sulphur in conidia uniformly labelled and in those incubated for a short time in medium with labelled sulphate but this difference was mainly due to differences in the relative amounts of incorporation into the soluble and residual fractions (Table 1).

Table 1. *Distribution of ^{35}S in Neurospora conidia*

Wild-type conidia (3·5 × 10^8) were incubated for 2 hr. at 30° with aeration. Uniformly labelled conidia were obtained from cultures of wild-type 74A grown for 5 days in a liquid Westergaards medium containing 5 μmole sulphur (as $^{35}SO_4$)/flask.

	% total sulphur in each fraction	
Fraction	Incubated 2 hr. with 0·3 μmole SO_4	Uniformly labelled
Soluble in:		
Cold trichloroacetic acid	27·5	10·0
Ethanol	1·5	4·3
Ethanol + ether	3·9	1·0
Warm trichloroacetic acid (TCA) (4 × at 70°)	2·4	3·8
Residue	64·6	80·8
% in last water wash before TCA	0·2	—

The identity of the material with the higher R_f value in these chromatograms was confirmed as being methionine, by the correspondence of the peaks of radio-activity (after phenol + water chromatography) with the spots of ninhydrin-reacting substance obtained by adding methionine or methionine sulphoxide (non-labelled) to the hydrolysates. The 'methionine spots' from phenol + water chromatograms of both wild-type and mutant preparations were eluted, evaporated and re-chromatographed in a butanol + acetic acid solvent, along with methionine and methionine sulphoxide standards. The 'methionine spot' from both mutant and wild-type preparations was a mixture of methionine and methionine sulphoxide showing that the mutant really did form labelled methionine.

Even though there was no visible germination (outgrowth production) by the wild-type after 2 hr. of incubation in low sulphur medium there was a definite synthesis of RNA (Table 2). Nucleic acid was determined in Neurospora by a method recently devised in our laboratory (Minagawa, Wagner & Strauss, 1959). Considering the well-established relationship between nucleic acid and protein synthesis (Cohn & Volkin, 1957), we believe that these data indicate that net protein synthesis occurred during a 2-hr incubation in low sulphur medium. It is obvious that net protein synthesis must have occurred in the course of producing a mycelial pad from an inoculum of conidia.

We were careful to check for traces of contamination by other organisms or by wild-type Neurospora in these experiments, since even a trace of methionine synthesis by contaminating organisms might account for our results. When incorporation into the mycelium was studied, parallel inoculations were made on minimal medium and a small piece of the mycelium produced was inoculated into minimal medium to make sure that the produced mycelium was still methionine-requiring. When conidial suspensions were incubated for short periods of time in the absence of methionine, portions of the suspension were inoculated into minimal medium at the start of the experiment and in addition a portion of the incubated material was examined with the oil-immersion lens for traces of bacterial growth.

Table 2. *Nucleic acid synthesis during short term incubation of wild-type 74 A*

Wild-type conidia ($3{\cdot}5 \times 10^8$) were incubated in 35 ml. modified minimal medium with aeration at 30°.

DNA = difference in optical density at 265–290 mμ; extract diluted to 10 ml.

RNA = difference in optical density 260–290 mμ; extract diluted to 100 ml. and optical density multiplied by 10.

Period of incubation (hr.)	SO_4 added (μmole)	DNA (265–290 mμ)	Increase (%)	RNA (260–290 mμ)	Increase (%)
0	—	0·147	—	2·04	—
		0·130		1·76	
2	0·0	0·163	17	2·74	34
		0·162		2·35	
2	0·15	0·159	17	3·17	61
		0·166		2·93	
2	1·2	0·163	17	3·86	103
				3·84	

After 15·5 hr. of incubation in a minimal medium the incorporation of $^{35}SO_4$ into the protein fraction of the methionine-requiring mutant was readily detected (Fig. 2). This radio-active material was identified as methionine by the method described above. Although labelled methionine was found in the protein fraction it was evident from an examination of the chromatogram tracings that the mutant contained less radio-active methionine relative to cysteine in its protein than the wild-type. In a separate experiment in which the two areas of the chromatograms of the protein hydrolysates were eluted, dried and counted, the methionine:cysteine ratio from the wild-type was 2·4 while the mutant ratio was 0·69. Incubation of the mutant in medium containing methionine gave a methionine:cysteine ratio of 1·19. Incubation of the mutant for shorter periods of time in minimal medium gave an even smaller methionine:cysteine ratio. Incubation of strain 584 for 9 hr. in minimal medium (with $^{35}SO_4$ of lower specific activity) gave a protein hydrolysate in which the cysteine was definitely labelled but in which the radio-active methionine area was not visible above the background. It should be emphasized that the free amino acid pool of wild-type conidia or of wild-type organism incubated under these conditions did not contain detectable methionine (see below). The relatively large difference between wild-type and mutant in the radio-active methionine:cysteine ratio was not seen in conidial preparations or in preparations from mutant and wild-type mycelium grown with methionine.

The free amino acid pool (extractable with warm 70 % (w/v) ethanol in water or with cold trichloroacetic acid) of the wild-type contained cystathionine and glutathionine (Table 3) but did not contain detectable methionine. The methionine-requiring mutant also contained cystathionine and glutathione but the relative amounts were different from the wild-type. The mutant, grown with methionine, did not contain detectable radio-active glutathione

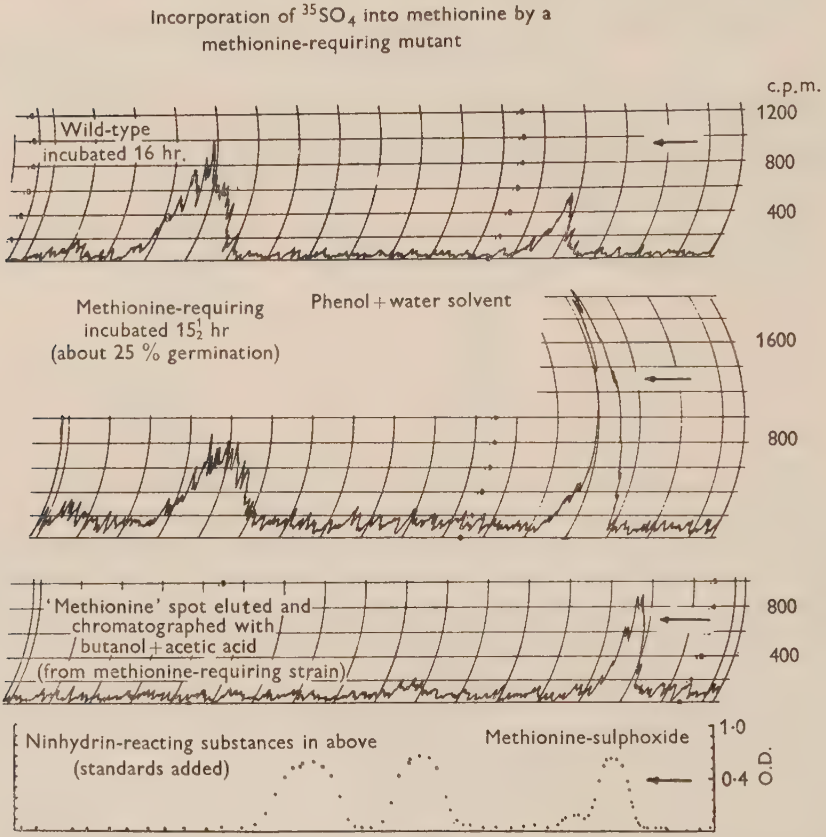

Fig. 2. Scanning patterns of the protein hydrolysate of conidia incubated overnight in minimal medium containing $^{35}SO_4$. About 2×10^8 non-labelled conidia were shaken overnight at 28° in 50 ml. modified minimal medium contained in 250 ml. Erlenmeyer flasks. The wild-type organism was incubated in media containing 3 μmole sulphate (10 μc.). The methionine-requiring mutant was incubated with 2 μmole sulphate (20 μc)/ 50 ml. After incubation the material was treated as described in the text. Arrows indicate the direction of flow of the chromatograms. The start and finish of the chromatograms are indicated by vertical lines. The methionine area was eluted from separate chromatograms, evaporated and rechromatographed in a butanol + acetic acid solvent mixture. Methionine and methionine sulphoxide (non-labelled) were added as standards. The ninhydrin-reacting material was recorded by plotting the optical density of the treated chromatogram as read with a 'Photovolt' densitometer. The position of the methionine sulphoxide spot as determined with separate standards is indicated on the figure.

after 2 days of growth, but did contain cystathionine. After 4 days the amount of cystathionine had decreased and glutathionine was visible. No sulphur-containing amino acids were detectable in the ethanolic extract of the mycelium after 6 days of incubation. No cystathionine was detected in the medium in which the methionine-requiring mutant was grown.

Table 3. *Fractionation of the fraction of mycelium soluble in 70 % (v/v) ethanol in water*

The numbers record the position of ninhydrin positive areas. Radio-active spots are italicized. Parentheses indicate ninhydrin-negative radio-active spots. The mycelium of the methionine-requiring mutants was harvested after 2, 4 or 6 days incubation in standing culture at 30° in 20 ml. minimal medium containing $^{35}SO_4$ and 0·5 μmole L-methionine. The mycelium was washed with water and dried *in vacuo*. Twelve mg. dried mycelium were extracted with boiling 70 % (v/v) ethanol in water for 30 min.; the extract was made up to 5 ml. and portions were chromatographed in phenol + water solvent. The wild type was grown in the absence of added methionine. Two-day wild-type cultures compared in dry weight to the 4-day mutant cultures described above. The area corresponding to cystathionine could not be distinguished from cysteine by paper chromatography. The extract was subjected to electrophoresis as described in the text. The identity of the various spots was checked by electrophoresis of superimposed standards. We assume that the radio-activity at the origin of the chromatograms was due to inorganic sulphate; the material at the finish of the chromatograms of the mutant after 4 days and the wild type after 2 days is unknown.

A. *Ninhydrin-positive and radio-active spots after phenol + water chromatography*

Strain	Period of incubation (days)	R_f values								
74A	2	*(4)*	*15*	*26*		39	46	57	71–91	*(96)*
584	2	*(2)*	—	*27*		35	47	56, 59		
584	4	*(2)*	*16*	*26*		39	45	56	72–89	*(99)*
584	6	—	—	—	29	34	45	52, 57		

R_f values of standards: methionine = 83, methionine sulphoxide = 78, cystathionine = 29, cysteine = 27, oxidized glutathione = 16.

B. *Ninhydrin positive and radio-active spots after electrophoresis*

Strain	Period of incubation (days)	Relative mobility (in % of the cysteine mobility)			
74A	2	—	*72*	*90*	*122*
74A (+ added glutathionine)	2	—	*62*	—	*118*
584	2	—	—	96	*120*
584	4	*0*	*71*	*90*	*126*
584 (+ added glutathione)	4	*0*	*69*		*118*

Relative mobility of standards: cystathionine = 122, methionine = 111, methionine sulphoxide = 100, cysteine = 100, reduced glutathione = 67.

DISCUSSION

We believe that the incorporation of ^{35}S from sulphate into methionine by the methionine-requiring mutant represents net synthesis of methionine. The growth data indicate that the mutant was eventually able to synthesize its protein (and therefore to synthesize methionine), since traces of growth were obtained in minimal medium after an extended growth lag and since a maximum yield of mycelium was obtained once growth had started. Incorporation of sulphur into methionine occurred during a period of protein synthesis as judged by the increase in nucleic acid (in the wild-type), by germination and by the production of a visible mycelium by the mutant in the presence of added non-labelled methionine. There are no exchange reactions known, at present, which might account for the reduction of sulphate and its binding to an organic molecule. We suspect that methionine is not synthesized by an alternative pathway although our data are not conclusive. If methionine is synthesized via cystathionine (Horowitz, 1950), cystathionine should accumulate at the start of growth, because of the genetic block. Since the block is 'leaky' the cystathionine should gradually disappear as the limited sulphur supply in the medium is used up and as methionine is formed, as we found. The synthesis of methionine by the mutant began rapidly and it seems probable that the use of $^{35}SO_4$ of high enough specific activity would permit detection of methionine synthesis after any period of incubation.

We think that the data contain an answer to the question: 'why, if the mutant can synthesize methionine, is there such a long lag before growth begins on minimal medium?'. The total mixture of wild-type protein (grown with $^{35}SO_4$ as the sole sulphur source) contains more methionine than cysteine. The ratio of ^{35}S-methionine: ^{35}S-cysteine in either the mycelium or the conidia of the mutant (grown in the presence of non-labelled methionine) is not very different from that of the wild-type grown under the same conditions. The ratio of protein methionine: cysteine formed from $^{35}SO_4$ by wild-type appears to be the same regardless of the duration of incubation or of the amount of sulphur in the medium. Although the wild-type ratio is relatively constant (judged from the chromatogram tracings) the behaviour of the mutant was quite different. The ratio of protein methionine:cysteine from $^{35}SO_4$ after 16 hr. was much lower than is characteristic of wild-type protein. Utilization of non-labelled stored methionine by the methionine-requiring mutant might account for these low ratios, since protein would be formed with this non-labelled methionine, but with cysteine produced from $^{35}SO_4$. However, there was no free methionine detectable in the amino-acid fraction of conidia or of mycelium. Glutathione, a peptide containing cysteine, was present. We suppose therefore that methionine was produced slowly by the normal pathway from the start of incubation and that the rate of methionine synthesis increased as growth proceeded.

The lower methionine:cysteine ratio in the newly formed mutant protein indicates that only a portion of the normal Neurospora proteins were made after incubation of the mutant for brief periods in minimal medium. Certain proteins, relatively rich in cysteine compared to methionine, must be able to compete more successfully for the limited supplies of newly-synthesized methionine made available by the mutant. We suppose that the mutant was not able to grow until those proteins, relatively richer in methionine than in cysteine, had been made. Growth is not possible from the start even though the required factor could be made.

In his study of the 'leaky' tryptophan-requiring mutants Bonner (1951) showed that seven out of the nine mutants tested made the substance they required. We suspect that this ability, demonstrated once again with the single methionine-requiring mutant reported herein, is fairly widespread. Many mutants, incubated on minimal medium, die off because of 'starvation death' (Strauss, 1958*a*). It is therefore not possible to determine whether they can make the factor they require, since the conidia (or mycelia) die as soon as starvation conditions arise because of exhaustion of growth factor. The phenomenon of 'leakage' described by Bonner may actually be typical for the class of nutritionally-deficient mutants.

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REFERENCES

Beadle, G. W. & Tatum, E. L. (1945). Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* **32**, 678.

Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, 484 pp. New York: Academic Press, Inc.

Bonner, D. M. (1951). Gene-enzyme relationships in Neurospora. *Cold. Spr. Harb. Symp. quant. Biol.* **16**, 143.

Bonner, D. M., Yanofsky, C. & Partridge, C. W. H. (1952). Incomplete genetic blocks in biochemical mutants of Neurospora. *Proc. nat. Acad. Sci., Wash.* **38**, 25.

Cohn, W. E. & Volkin, E. (1957). The nucleic acids. *Ann. Rev. Biochem.* **26**, 491.

Horowitz, N. H. (1950). Biochemical genetics of Neurospora. *Advanc. Gentet.* **3**, 33.

Minagawa, T., Wagner, B. & Strauss, B. (1959). The nucleic acid content of *Neurospora crassa. Arch. Biochem. Biophys.* (in the Press).

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). Studies of biosynthesis in *Escherichia coli. Publ. Carneg. Instn*, No. 607.

Strauss, B. S., Vaharu, T. & Frickey, P. (1956). Studies on the lethal effect of incorporated P^{32} and S^{35} in *Neurospora crassa. Rad. Res.* **5**, 25.

Strauss, B. S. (1958*a*). Cell death and 'unbalanced growth' in Neurospora. *J. gen. Microbiol.* **18**, 658.

Strauss, B. S. (1958*b*). Effect of the distribution of incorporated sulfur on the lethal effect of incorporated $^{35}SO_4$. *Proc. 10th Int. Congr. Genet.* (in the Press).

Woodward, V. W., DeZeeuw, J. R. & Srb, A. M. (1954). The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia followed by filtration and selective plating. *Proc. nat. Acad. Sci., Wash.* **40**, 192.

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FAINE, S. (1959). *J. gen. Microbiol.* **20**, 246–251.

Iron as a Growth Requirement for Pathogenic Leptospira

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SUMMARY: Virulent and avirulent strains of *Leptospira icterohaemorrhagiae* did not grow from inocula of fewer than 10^7 organisms/ml. in Korthof medium, but did grow when $FeCl_3$ or preparations containing iron porphyrins were added. With added $FeCl_3$ or haematin growth was somewhat slower than when the iron porphyrin-containing preparations were added.

Many authorities have recommended that the serum essential in culture media for pathogenic leptospires should contain small quantities of haemolysed erythrocytes, or that a solution of laked erythrocytes should be added as a 'source of haemoglobin' (Alston & Broom, 1958; Wolff, 1954). There has been no agreement about the need for haemoglobin nor suggestions about its possible functions. This report describes experiments made with Korthof medium which did not support growth of Leptospira but did so when $FeCl_3$, haematin or preparations containing iron-porphyrin compounds were added.

METHODS

Organisms. The strains used were *Leptospira icterohaemorrhagiae* (Jackson) avirulent for guinea-pigs, and *L. icterohaemorrhagiae* (Field), virulent and in its 5th laboratory subculture since guinea-pig passage. They were obtained in 1953 from Dr J. C. Broom (Leptospira Reference Laboratory, Wellcome Laboratories of Tropical Medicine, London) and maintained in modified Korthof medium. Incubation was at 30° .

Counting method. A direct counting method (Faine, 1957) was used.

Culture medium. Broom's modification (Alston & Broom, 1958) of Korthof's medium (Korthof, 1932) was used but with the substitution of Neopeptone (Difco) for the Witte peptone recommended. All other ingredients were 'pure' or of analytical grade. The batch of medium used in these studies, designated K11, was prepared to contain 10% (v/v) pooled rabbit serum obtained by heart puncture, care being taken to avoid haemolysis. The medium was sterilized by filtration through a Seitz E.K. filter-pad; it contained 40 μg. Fe/100 ml. This concentration of Fe may be compared with that in a sample of a complete medium with added 'Hb solution' (see below) adequate for growth and which contained *c.* 190 μg. iron/100 ml.

Sources of iron and porphyrins. Solutions containing haemoglobin ('haemoglobin solution' = Hb solution in the sequel) were prepared by repeatedly freezing and thawing washed packed rabbit erythrocytes, and were sterilized

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by Seitz filtration. The haemoglobin content of this material was measured photometrically and dilutions were prepared from a stock standard solution. Solutions containing derivatives of haemoglobin were prepared from Hb solution as follows: containing carboxyhaemoglobin (COHb) by bubbling coal-gas through; containing methaemoglobin (MetHb) by adding 0·2 % (w/v) potassium ferricyanide; containing 'boiled Hb' by boiling for 30 min.; containing 'heated Hb' by heating at 56° for 30 min. Haematin was prepared by adding NaOH to crystalline ox haemin. $FeCl_3$ was diluted from 'Analar' 60 % (w/v) solution. Water distilled twice from glass was used to rinse glassware and for dilutions.

RESULTS

The need for iron or iron porphyrin compounds

It was found that the leptospires used would not grow well in medium K11, but that this supported luxuriant growth when a little Hb solution was added. In an experiment to find substitutes for the Hb solution, 16 ml. of medium K11 were inoculated with 0·5 ml. of a 3-day culture of *Leptospira icterohaemorrhagiae* (Jackson); the final concentration was 10^6 organisms/ml. The inoculum was contained in 1/30 the volume of medium to be inoculated. The inoculated medium was distributed in duplicate 1 ml. volumes. To each of two control tubes was added 0·01 ml. H_2O. To each of other pairs was added 0·01 ml. of a *c.* 0·01 M solution containing one of the following: Hb, boiled Hb, heated Hb, COHb, MetHb, haematin or $FeCl_3$. The organisms were counted in each tube after incubation for 4, 7 and 18 days at 30° (Table 1). Growth was slower with added haematin or $FeCl_3$ than with the other supplements. Similar results were obtained with two virulent strains of *L. icterohaemorrhagiae*. Growth in medium K11 was not enhanced by heating it at 56° for 30 min., nor by adding 1, 10 or 100 μg. cyanocobalamin/ml., or 10^7 boiled washed leptospires/ml.

Table 1. *Growth-promoting effect of* $FeCl_3$ *and material containing haemoglobin and certain derivatives*

The medium K11 (iron and porphyrin deficient) was inoculated with 10^6 leptospires/ml.

Supplements were added to *c.* 10^{-4} M final concentration, in duplicate series. The numbers of leptospires given are average of duplicates.

	Period of incubation (days)		
	4	7	18
	Leptospires (millions)/ml.		
Medium + H_2O	2	7	1
Medium + Hb solution	110	309	167
Medium + heated Hb solution	99	295	—
Medium + COHb solution	145	324	—
Medium + MetHb solution	130	—	—
Medium + haematin	34	232	230
Medium + boiled Hb solution	82	164	—
Medium + $FeCl_3$	44	286	125

Optimum concentration of added iron or other supplement

The optimum concentration of Hb solution or Fe to promote growth was found by adding serially diluted solutions to inoculated K11 medium. Duplicate tenfold serial dilutions of Hb solution and of $FeCl_3$ containing the equivalent of 10^{-2} M-Fe were made in sterile double glass-distilled water. To tubes containing 0·98 ml. of medium K11 were added 0·01 ml. of the appropriate dilution of Hb solution or $FeCl_3$ and 0·01 ml. of a 1/10 dilution of a 4-day culture of *Leptospira icterohaemorrhagiae*. This resulted in a range of Fe concentrations from 10^{-4} to 10^{-8} M (as Hb solution or $FeCl_3$). Each tube contained in 1·0 ml. of medium 10^4 leptospires and 10^{-3} ml. of medium carried over from the inoculating culture. Control tubes contained supplements of double glass-distilled water alone. The organisms were counted at intervals (Table 2), but not all tubes were counted at the same time. The optimum supplement concentration was equivalent to *c.* 10^{-5} M-Fe as haemoglobin in the Hb solution or as $FeCl_3$. Growth was faster with added Hb solution than with added $FeCl_3$ at equivalent Fe concentrations. There was a threshold concentration of added iron which was just above the concentration in unsupplemented medium.

Effect of size of inoculum

It has frequently been stated that a large inoculum of 1/6–1/10 volume should be used in routine subculture of leptospires (Wolff, 1954; Alston & Broom, 1958; Rimpau, 1950; Wiesmann, 1952); this would be equivalent to *c.* 10^7 organisms/ml. Inocula of *c.* 100 washed or unwashed organisms/ml. grew typically and satisfactorily in Korthof medium. The function of a large inoculum would seem to be to supply iron or some iron-porphyrin contained in the transferred leptospires or medium. Half a washed suspension of organisms from a 5-day culture in adequate medium was serially 10-fold diluted in duplicate 1 ml. volumes of medium K11. The other half was similarly diluted in medium K11 containing the equivalent of 10^{-5} M haemoglobin as Hb solution. Final concentrations of inoculum were 10^8 to 10^2 organisms/ml. Growth up to *c.* 10^8 leptospires/ml. occurred in 2–13 days in all media with added Hb solution. From one to four generations occurred in *c.* 5 days in medium K11 without added Hb solution and inoculated with 10^8 or 10^7 organisms/ml., but there was no growth in tubes inoculated with 10^6 organisms or less/ml., within 20 days. In a similar experiment boiled organisms to 5×10^7/ml. added to each tube of K11 medium prolonged survival of inocula of 10^6 organisms/ml. as compared with the controls, but did not promote growth nor alter the results in other tubes.

DISCUSSION

There has hitherto been little or no evidence that haemoglobin, a constituent of many recipes for culture media for Leptospira, is an essential nutrient requirement for these organisms; nor has there been any indication of its possible functions. Unless special precautions are taken, at least traces of

Table 2. *Effect of different concentrations of* $FeCl_3$ *or* Hb *solution* (*measured as haemoglobin*) *supplements on multiplication of* Leptospira icterohaemorrhagiae.

Cultures in deficient medium supplemented with 10^{-4} to 10^{-8}M-Fe measured as haemoglobin in Hb solution or $FeCl_3$. Inoculum 10^4 leptospires/ml.; carry-over 10^{-3} ml. medium.

	Added iron (molarity)										
	As haemoglobin in Hb solution					As $FeCl_3$					
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
	Total μg. Fe/100 ml. medium										
Period of incubation (days)	600	95	45	40	40	600	95	45	40	40	No added material H_2O blank
	No. leptospires (millions)/ml.										
3	0·007	0·005	0·002	—	—	—	0·007	0·005	0·007	0·002	0·001
5·5	0·12	0·25	—	—	0·002	—	—	—	—	—	—
7	10	20	0·005	—	—	0·015	0·33	0·005	0·005	—	—
9	23	192	0·015	0·005	0·01	0·012	0·54	0·01	0·03	0·02	—
12	143	116	0·04	0·03	0·05	0·03	9·9	0·2	0·2	0·02	0·1
16	75	162	275	—	0·6	0·6	2·6	0·65	0·6	0·03	0·25
18	—	—	—	0·76	50	0·12	43·5	1·0	1·5	0·02	2·1
23	80	69	120	—	18	5·0	151	5·0	3·3	0·02	0·5
26	—	—	120	<0·001	7·0	6·0	80	7·0	0·75	<0·001	3·0

haemoglobin are probably present in the serum which is generally used in all media for Leptospira. The original Korthof medium (Korthof, 1932) did not recommend the addition of laked erythrocytes, but Broom's modification does (Alston & Broom, 1958). Babudieri (1943) found that adding laked erythrocytes made no difference to the ability of Leptospira to survive for long periods in culture media. Czekalowski, McLeod & Rodican (1953) were unable to show catalase or unequivocal oxidase activity in their organisms and later suggested that media containing laked erythrocytes were better than others because this material supplied catalase (Czekalowski, McLeod & Rodican 1954). Fulton & Spooner (1956) showed the spectrum of cytochrome *c*, and Faine (1958) demonstrated oxidase but not catalase activity, in *Leptospira icterohaemorrhagiae* (Jackson); the 'Field' strain and many other serotypes of Leptospira contained oxidases as well as catalase. Haemoglobin may be a source of Fe and porphyrin for the building of these enzymes. The Leptospira used in the present experiments grew very slowly and to a limited extent unless Fe, or material containing iron porphyrins, were added. The faster growth rate with Hb solution and some derivatives of it than with haematin or Fe suggested that Leptospira may synthesize more slowly from medium constituents + Fe those cellular components, presumably Fe porphyrin enzymes, for which the iron porphyrin compounds from laked erythrocytes are more effective precursors. Haematin was a constituent of the defined and partially defined media proposed by Savino & Rennella (1943). Tap water, recommended for use in many commonly used media, such as the original medium of Vervoort-Schüffner (see van Thiel, 1948) would provide significant amounts of Fe. On the other hand, Fe might be removed by chelation with phosphates during the preparation of these media. Traces of Fe might be introduced by filtration through filter pads (Hunter, 1943). The optimum Fe supplement of 10^{-5} M corresponds approximately to the concentration of haemoglobin in modified Korthof medium (Alston & Broom, 1958) supplemented with Hb solution as used here.

A heavy inoculum of culture equivalent to 1/6–1/10 of the volume of medium to be inoculated has been recommended and used by many workers (Alston & Broom, 1958; Wolff, 1954). Enough Leptospira organisms might thus be transferred for growth to become visible after only 4–5 divisions, which is the degree of growth obtained in medium K11 inoculated with 10^7 or more washed organisms/ml. Personal experience shows that *c.* $1–5 \times 10^7$ leptospires/ml. can be seen with the naked eye in tubes or bottles of 10–20 mm. internal diameter. Thus iron or iron porphyrin deficiency may not be important when media are very heavily inoculated. Conversely, large inocula are not necessary for growth in media made adequate by addition of iron or iron porphyrin-containing material because 10^2 washed leptospires/ml. grew to usual numbers, *c.* 10^8/ml., in medium K11 supplemented with Hb solution.

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REFERENCES

ALSTON, J. M. & BROOM, J. C. (1958). *Leptospirosis in Man and Animals.* Edinburgh: E. and S. Livingstone Ltd.

BABUDIERI, B. (1943). Das Überleben von Leptospiren auf Serum- und Blutnährböden. *Zbl. Bakt.* (1 *Abt. Orig.*), **150**, 243.

CZEKALOWSKI, J. W., McLEOD, J. W. & RODICAN, J. (1953). The growth and respiration of Leptospira in solid or semi-solid media with special reference to Dinger's phenomenon. *Brit. J. exp. Path.* **34**, 588.

CZEKALOWSKI, J. W., McLEOD, J. W. & RODICAN, J. (1954). Observations on the nutrition of Leptospira based on the development of Dinger's phenomenon. *J. gen. Microbiol.* **10**, 199.

FAINE, S. (1957). Virulence in leptospira. I. Reactions of guinea-pigs to experimental infection with *Leptospira icterohaemorrhagiae. Brit. J. exp. Path.* **38**, 1.

FAINE, S. (1958). Respiratory enzyme activity, and the need for haemoglobin in cultures of pathogenic leptospira. *Proc. Univ. Otago Med. Sch.* **36**, 27.

FULTON, J. D. & SPOONER, D. F. (1956). The metabolism of *Leptospira icterohaemorrhagiae* in vitro. *Exp. Parasit.* **5**, 154.

HUNTER, G. J. E. (1943). Growth-promoting substances from bacteriological filter pads. *Biochem. J.* **37**, 577.

KORTHOF, G. (1932). Experimentelles Schlammfieber beim Menschen. *Zbl. Bakt.* (1. *Abt. Orig.*), **125**, 429.

RIMPAU, W. (1950). *Die Leptospirose.* München: Urban and Schwarzenberg.

SAVINO, E. & RENNELLA, E. (1943). El cultivo de la *Leptospira icterohaemorrhagiae* Inada e Ido, 1915. IV. Cultivo en medio sintético y semisintético. *Rev. Inst. bact. Malbran.* **11**, 322. (Abstract by Broom, J. C. (1944). *Bull. Hyg.* **19**, 484.)

VAN THIEL, P. H. (1948). *The Leptospiroses.* Leiden: Universitaire Pers Leiden.

WIESMANN, E. (1952). In *Leptospirosen*, by Gsell, O. and Wiesmann, E. Bern: H. Huber.

WOLFF, J. W. (1954). *The Laboratory Diagnosis of Leptospirosis.* Springfield, Ohio: C. C. Thomas.

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Adams, M. E. & Postgate, J. R. (1959). *J. gen. Microbiol.* **20**, 252–257

A New Sulphate-Reducing Vibrio

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SUMMARY: An obligately anaerobic sulphate-reducing vibrio (NCIB 8382) isolated from soil from Singapore differs from known species of sulphate-reducing bacteria. The organism is relatively large, with one or two flagella, Gram-negative, lacks hydrogenase and pigments characteristic of *Desulphovibrio desulphuricans*, though an insoluble cytochrome is present. It grows with lactate or pyruvate, but is unable to metabolize acetate, propionate or butyrate; it reduces sulphate, sulphite or thiosulphate but not tetrathionate. Sporulation is rare but has been observed. The name *Desulphovibrio orientis* is proposed for this organism.

The number of different organisms believed to be capable of dissimilatory sulphate reduction is small. Baars (1930) devoted the opening chapter of his thesis to a survey of organisms reported earlier and concluded that all the better-authenticated ones corresponded to Beijerinck's *Spirillum desulphuricans* (Beijerinck, 1895) or the other two species: *S. aestuarii* (van Delden, 1903); *S. thermodesulphuricans* (Elion, 1925). (The generic name of this group has at various times been *Spirillum*, *Microspira*, *Vibrio*, *Sporovibrio*; at present *Desulphovibrio* is used most widely.) Baars's thesis included evidence that the species *aestuarii* and *thermodesulphuricans* were variants of the type species *desulphuricans*, which was able to utilize a variety of carbon sources but not butyrate, propionate or acetate. Baars introduced a new species *rubentschikii*, which was distinguished by being able to utilize these fatty acid salts, and a variant, *rubentschikii* var. *anomalous* able to utilize butyrate or propionate but not acetate. Though Baars's views were disputed in certain details (e.g. ZoBell, 1948), the opinion that dissimilatory sulphate-reducing bacteria were confined to the one genus now called *Desulphovibrio* was widely accepted for about a quarter of a century. Occasional reports were made of sulphate-reducing organisms outside the genus *Desulphovibrio*: Hvid-Hansen's (1951) *Desulphoristella* was lost soon after isolation, and Prévot's sulphate-reducing clostridia (Prévot, 1948) lost their ability to reduce sulphate on transfer in laboratory media. Campbell, Frank & Hall (1957) showed that the species *thermodesulphuricans* is in fact *Clostridium nigrificans* and that the evidence obtained by Baars and later by Starkey (1938), indicating that it was a variant of *desulphuricans*, must have been wrongly interpreted. This observation required the re-classification of an existing type but not the introduction of a new one.

The present paper reports the isolation of a species of dissimilatory sulphate-reducing bacterium which does not fit into any pre-existing species, and for which the name *Desulphovibrio orientis* is proposed.

ISOLATION OF THE STRAIN

A soil sample was received at this laboratory from the neighbourhood of a rising main at Sungei Whampoa, Rangoon Road, Singapore. An enrichment culture prepared by the procedure of Butlin, Adams & Thomas (1949) showed considerable blackening at 30° but none at 55°. The enrichment culture at 30° contained no forms typical of *Desulphovibrio desulphuricans*; a considerable population of large slightly curved rods, often paired, was present; a few spore-forming rods were also seen. Colony isolation in medium A of Butlin *et al.* (1949) ultimately yielded a homogeneous culture of the curved organisms; no aerobes were detected by plating out on glucose peptone agar and no anaerobes apart from sulphate-reducing bacteria were detected in deep nutrient agar (Postgate, 1953). The organism grew erratically in either medium C or in medium A of Butlin *et al.* (1949); when these media were supplemented with thiolacetate (0·01–0·02 %, w/v) the organism grew regularly. It has been freeze-dried and deposited with the National Collection of Industrial Bacteria (NCIB) as organism no. 8382.

Distribution. Enrichment cultures made from two other soil samples from the same site yielded the new type of sulphate-reducing organism but typical *Desulphovibrio desulphuricans* was not observed. One soil sample yielded thermophilic sulphate-reducing bacteria typical of *Clostridium nigrificans*. A fourth sample from Ulu Pandan, some 4 miles away, yielded the new type and from it a second pure culture was isolated (NCIB 8445). Samples taken three years later from the original sites still yield the new organisms, so did one from the McRitchie reservoir five miles away. Samples from neighbouring watercourses, cultivated soil and from the sea at Singapore yielded ordinary *D. desulphuricans*. Enrichment cultures made from soil near a termite comb in Kanpur, India, and from a marshy desert in West India also yielded the new organism.

Morphology. The organism was a fat rod, often slightly curved, sometimes paired or in short chains; mean dimensions: $4{\cdot}85 \times 1{\cdot}4\,\mu$. The organisms were uniformly Gram-negative and staining procedures provided no evidence for capsules. Non-progressive 'twisting and turning' motion was sometimes seen and was particularly marked in preparations taken from colonies in agar media. Cultures 5 or more days after growth contained a considerable number of organisms which showed granulated cytoplasm; forms with a single central or near-central granule were sometimes seen.

Preparations stained with Casares-Gil stain showed many organisms with one or two flagella, and forms which appeared peritrichous were often seen. Dr A. R. Prévot kindly examined cultures for us and came to the following conclusions: '...la forme la plus fréquent est le cil unique polaire ou parapolaire. Puis vient en deuxième position la ciliature bipolaire, enfin moins fréquente la ciliature en apparence péritriche 3 à 4 cils. Mais si on analyse minutieusement les formes à 3 ou 4 cils on s'aperçoit que ce sont des diplovibrions ou des chainettes de vibrions.'

Cultivation. The organism showed growth after 36–72 hr. when inoculated into the media mentioned above, when thiolacetate was present. Cysteine, which has been used for poising the E_h value of cultures of *Desulphovibrio desulphuricans* (Grossman & Postgate, 1953), was not as satisfactory in promoting consistent growth of the strain as was thiolacetate; Na_2S could be used in place of thiolacetate but was not desirable when excess ferrous ion was present in the medium. Cultures were incubated at 30° in McIntosh & Fildes anaerobic jars filled with H_2 99 % (v/v) + CO_2 1 % (v/v), in wholly filled and stoppered bottles, or in stoppered vessels made anaerobic with a cottonwool plug containing pyrogallol (sat.) + Na_2CO_3 (sat.). The pH range in which most rapid growth in medium C occurred was 6·5–7·0 (± 0·2); growth did not occur outside the limits pH 6·0 and 8·0.

The final yield of organisms in medium C was equivalent to 200–250 μg. dry wt. organisms/ml. (about two-thirds of the yield of *Desulphovibrio desulphuricans* in a similar medium). When the yeast extract (0·1 %, w/v) was omitted, only 20–40 μg. organism/ml. was obtained; yeast extract could be replaced by Tryptone (Difco; 0·1, w/v) or acid-hydrolysed casein (Oxoid; 0·1, w/v) but not by the ash of yeast extract (added equiv. 0·1 %, w/v, original material).

Sulphate reduction. Cultures in medium C + thiolacetate were analysed iodometrically for sulphide; in 5 days the sulphide concentration was 3·2 μmole S″/ml. (108 p.p.m. H_2S). An 8-day culture was analysed for sulphide, lactate (Neish, 1950) and acetate (Grossman & Postgate, 1955). It contained: 8·6 μmole S″/ml. (292 p.p.m. H_2S); 19·5 μmole lactate/ml. (content of uninoculated medium: 35 μmole/ml., hence 15·5 μmole/ml. utilized); 17 μmole acetate/ml. These figures are reasonably consistent with the equation:

$$2CH_3CHOH.COOH + Na_2SO_4 = Na_2S + CH_3COOH + CO_2 + 2H_2O,$$

which is well established for *Desulphovibrio desulphuricans* and thermophilic sulphate-reducing bacteria. Growth in medium C took place when the sulphate was replaced by Na_2SO_3 or $Na_2S_2O_3$, but not $Na_2S_4O_6$.

Utilization of carbon sources. This question was not studied exhaustively and only compounds relevant to the classification of known sulphate-reducing species were tested. The organism did not grow when acetate, propionate or butyrate replaced lactate in media A or C. When pyruvate replaced lactate in medium C the organism grew, but to no greater extent than with lactate (Senez, 1951, showed improved growth of marine *Desulphovibrio desulphuricans* in media having the formula of medium C but containing pyruvate in place of lactate). In the absence of sulphate, pyruvate did not permit growth; with certain strains of *D. desulphuricans* pyruvate will support ‘sulphate-free’ growth (Postgate, 1952).

Nitrate reduction. This was not detected by the micro-method of Clarke & Cowan (1952); a parallel control with *Chromobacterium prodigiosum* showed nitrate reduction.

Gelatinase formation. Gelatinase was not detected by Clarke & Cowan’s (1952) procedure, though it was present in a control suspension of *Chromobacterium prodigiosum*.

Cytochromes and other pigments. Heavy suspensions of organisms (*c.* 35 mg. dry wt./ml.) showed a faint broad absorption band at *c.* 557 mμ when observed in a Hartridge reversion spectroscope; this band disappeared on shaking in air. Treatment with alkaline pyridine and dithionite gave a 'haemochromogen' band at *c.* 554 mμ, suggesting the presence of a protohaem derivative. No haemoprotein appeared in solution when organisms were boiled, treated with a cationic detergent, or when acetone-dried organisms were extracted with phosphate buffer. This behaviour is not characteristic of cytochrome c_3 (the cytochrome of *Desulphovibrio desulphuricans*; see Postgate, 1956), but it implies the presence of relatively small amounts of an insoluble cytochrome-like pigment.

No absorption bands in the neighbourhood of 630 mμ were seen, and suspensions of organisms did not fluoresce red when treated with alkali and exposed to light at 365 mμ; hence the pigment desulphoviridin, characteristic of *Desulphovibrio desulphuricans* (Postgate, 1956), was absent.

Hydrogenase. Suspensions of the organism (equiv. *c.* 0·8 mg. dry wt. organism/ml.) did not decolorize methylene blue (0·05 μM) faster than controls (*c.* 3 hr. at 37°) in Thunberg tubes at pH 7·0. A similar suspension did not absorb H_2 in Warburg manometers when incubated for 1 hr. at 37° with methyl violet. This dyestuff is among the more sensitive indicators of hydrogenase (King & Winfield, 1955; Littlewood & Postgate, 1956). Suspensions of equiv. *c.* 8 mg. dry wt. organism/ml. did not release gas from pyruvate in similar conditions (*Desulphovibrio desulphuricans* dismutes pyruvate to products including H_2, CO_2 and acetate; Senez, 1954). These observations indicate that hydrogenase was absent.

Sporulation. Efforts were made to observe spores in cultures of the new organism, but the morphological picture was confused by the frequent presence of granular forms. Cultures were inspected at ages ranging from 24 hr. to several months; crushed colonies in agar (which show sporulation of *Clostridium nigrificans* well) were inspected, and so were cultures grown with 100 μg. $MnCl_2.4H_2O$/ml. (which induces spore formation in *C. nigrificans* according to a personal communication from Professor O. B. Williams). None of these expedients induced unequivocal sporulation, nor did culture with 1·5 % Tryptone (Hitzman, Halvorson & Ukita, 1957), exposure to air, glucose (1 %, w/v) or distilled water, nor yet acclimatization to growth at 42°. Cultures of the organism were rendered sterile by heating to 80° for 1 min. Dr A. R. Prévot, however, reported sporulation of this strain, and said: 'La sporulation apparait rarement; mais il s'agit d'une spore presque centrale, plus large que le vibrion, ne ressemblant en rien aux spores clostridiennes de *C. nigrificans*. Nous ignorons tout du déterminisme de cette sporulation. Mais une fois de plus j'ai observé que, quand elle apparait, elle confère à la culture ou elle se trouve une légère thermorésistance: 5 minutes à 80° alors que les cultures non-sporulées sont tuées à 70°' (Dr A. R. Prévot, personal communication). We have since been able to repeat all of Dr Prévot's observations on sporulation and on three occasions have observed *c.* 90 % sporulation in ordinary cultures.

Temperature range. The organism was isolated at 30°; it grew readily at 37° though not above 40°. By successive subculture with temperature increments of 1° it was 'trained' to grow at 42°. It did not then grow at 42·5°; adaptation to a truly thermophilic habit did not occur. During training swollen forms of the vegetative organism appeared but such morphological changes were only temporary.

DISCUSSION

The strain described here is physiologically and morphologically different from *Desulphovibrio desulphuricans* and *D. aestuarii.* Its inability to oxidize acetate, propionate or butyrate distinguishes it from *D. rubentschikii* and from *D. rubentschikii* var. *anomalous*; its mesophilic habit and failure to adapt to thermophily, together with its different morphology, distinguishes it from *Clostridium nigrificans.* It differs from Hvid-Hansen's *Desulphoristella* in its motility, its larger size, and its inability to utilize acetate or gaseous H_2. Its morphology and physiology, while not necessarily implying a phylogenetic relationship to members of the genus *Desulphovibrio*, nevertheless provide reasons for its incorporation in the same genus. In view of its origin far to the east of the meridian of Greenwich we propose the specific designation *orientis*; in the classification used by Prévot (1957) it would become *Sporovibrio orientis.* A formal description of the species follows.

Desulphovibrio orientis n.sp.

Curved rods, sometimes paired or in short chains, *c.* $4 \cdot 8 \times 1 \cdot 5\,\mu$, spores rare, central or paracentral. One or two polar or near-polar flagella.

Motile (non-progressive), non-capsulate, Gram-negative.

Obligate anaerobe, optimum temperature range 30–37°.

Grows slowly on specialized media containing lactate, sulphate and thiolacetate; utilizes pyruvate in place of lactate, but not butyrate propionate or acetate. Reduces sulphates to sulphide; forms black colonies in agar media containing ferrous salts.

Nitrate not reduced.

Gelatin not hydrolysed.

Contains a cytochrome pigment; desulphoviridin absent.

Hydrogenase absent.

Isolated from a soil sample from Singapore.

Subculture deposited with the National Collection of Industrial Bacteria, National Chemical Laboratory, Teddington, England; strain no. 8382.

We are indebted to Dr A. R. Prévot for examining the organism and for giving us his views on its nomenclature; he is not entirely in agreement with us about the broader nomenclature of the sulphate-reducing bacteria and we accept full responsibility for the final decision. We are also indebted to Mr E. Carlos of the Singapore Municipality and Dr J. J. Bagchi of the Indian Department of Defence for providing samples for examination. This paper is published by permission of the Director, National Chemical Laboratory.

REFERENCES

BAARS, J. K. (1930). *Over sulfaatreductie door bacterien.* Dissertation; Meinema: Delft.

BEIJERINCK, M. W. (1895). Über *Spirillum desulfuricans* als Ursache von Sulfatreduktion. *Zbl. Bakt.* (2. *Abt.*) 1, 1, 49, 104.

BUTLIN, K. R., ADAMS, M. E. & THOMAS, M. (1949). The isolation and cultivation of sulphate-reducing bacteria. *J. gen. Microbiol.* 3, 46.

CAMPBELL, L. L., FRANK, H. A. & HALL, E. R. (1957). Studies on thermophilic sulphate-reducing bacteria. I. Identification of *Sporovibrio desulfuricans* as *Clostridium nigrificans. J. Bact.* 73, 516.

CLARKE, P. H. & COWAN, S. T. (1952). Biochemical methods for bacteriology. *J. gen. Microbiol.* 6, 187.

DELDEN, A. VAN (1903). Beitrag zur Kentniss der Sulfatreduktion durch Bakterien. *Zbl. Bakt.* (2. *Abt.*), 11, 81, 113.

ELION, L. (1925). A thermophilic sulphate-reducing bacterium. *Zbl. Bakt.* (2. *Abt.*), 63, 58.

GROSSMAN, J. P. & POSTGATE, J. R. (1953). Cultivation of sulphate-reducing bacteria. *Nature, Lond.* 171, 600.

GROSSMAN, J. P. & POSTGATE, J. R. (1955). The metabolism of malate and certain other compounds by *Desulphovibrio desulphuricans. J. gen. Microbiol.* 12, 429.

HITZMAN, D. O., HALVORSON, H. O. & UKITA, T. (1957). Requirements for production and germination of spores of anaerobic bacteria. *J. Bact.* 74, 1.

HVID-HANSEN, N. (1951). Sulphate-reducing and hydrocarbon-producing bacteria in ground-water. *Acta path. microbiol. scand.* 29, 314.

KING, N. K. & WINFIELD, M. E. (1955). The assay of soluble hydrogenase. *Biochem. biophys. Acta,* 18, 431.

LITTLEWOOD, D. & POSTGATE, J. R. (1956). Substrate inhibition of hydrogenase enhanced by sodium chloride. *Biochim. biophys. Acta,* 20, 399.

NEISH, A. C. (1950). Analytical methods for bacterial fermentation. *Rep. nat. Res. Coun. Can.* no. 46–8–3. Saskatoon.

POSTGATE, J. R. (1952). Growth of sulphate-reducing bacteria in sulphate-free media. *Research,* 5, 189.

POSTGATE, J. R. (1953). On the nutrition of *Desulphovibrio desulphuricans*: a correction. *J. gen. Microbiol.* 9, 440.

POSTGATE, J. R. (1956). Cytochrome c_3 and desulphoviridin; pigments of the anaerobe *Desulphovibrio desulphuricans. J. gen. Microbiol.* 14, 545.

PRÉVOT, A. R. (1948). Recherches sur la réduction des sulfates et des sulfites minéraux par les bactéries anaérobies. *Ann. Inst. Pasteur,* 75, 571.

PRÉVOT, A. R. (1957). *Manuel de classification et de détermination des bactéries anaérobies,* 3rd ed. Paris: Masson and Co.

SENEZ, J. C. (1951). Étude comparative de la croissance de *Sporovibrio desulfuricans* sur pyruvate et sur lactate de soude. *Ann. Inst. Pasteur,* 80, 395.

SENEZ, J. C. (1954). Fermentation de l'acide pyruvique et des acides dicarboxyliques par les bactéries anaérobies sulfato-réductrices. *Bull. Soc. Chim. biol. Paris,* 36, 541.

STARKEY, R. L. (1938). A study of spore formation and other morphological characteristics of *Vibrio desulfuricans. Arch. Mikrobiol.* 9, 268.

ZOBELL, C. E. (1948). In Bergey's *Manual of Determinative Bacteriology* (ed. Breed, Murray, & Hitchens), 6th ed. p. 207. Baltimore, U.S.A.: Williams and Wilkins.

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The Nature of the Capsular Polysaccharides of the Dextran-Producing Organisms *Leuconostoc mesenteroides, L. dextranicum* and *Streptococcus bovis*

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SUMMARY: The true capsular polysaccharide of *Streptococcus bovis* contains galactose, glucose, rhamnose and an uronic acid as constituent sugars, whereas those of *Leuconostoc mesenteroides* and *L. dextranicum* contain only galactose and glucose in about equal proportions. Contamination of the capsule with dextran, not easily removed by simple washing of the harvested organisms, may occur with all species when grown in sucrose or in glucose media. This contamination is minimized when a glucose medium is inoculated with a glucose culture many times removed from a sucrose-containing medium.

Since *Streptococcus bovis*, like *Leuconostoc mesenteroides* and *L. dextranicum*, is an encapsulated organism and can produce large yields of dextran in suitable liquid sucrose media (Bailey & Oxford, 1958*a, b*), it seemed of interest to isolate and compare the chemical composition of their respective capsular polysaccharides. By the term 'capsular polysaccharide' is here meant the non-dialysable soluble carbohydrate extractable from the well-washed organisms by the mild sodium acetate + acetic acid procedure of Hobson & Macpherson (1954). These authors have already examined the capsular material so extracted from a rumen streptococcus very similar to *S. bovis* in fermentation reactions. We were particularly interested in determining whether the capsular polysaccharides produced from sucrose (the only known dextran precursor) did in fact contain dextran or were in any way different from those produced from glucose and other fermentable sugars. It has been possible to test for the presence of dextran in the capsular extract by using a dextranase produced by *Lactobacillus bifidus* (Bailey & Clarke, 1959). This enzyme produces a distinctive and unusual mixture of *iso*maltodextrins from dextran. We here present evidence that the true capsular polysaccharides of *Leuconostoc* species and of *S. bovis* are chemically different and that dextran, although often present in such material, is to be regarded in each instance as a contaminant rather than as the true capsular polysaccharide.

METHODS

Organisms. Six rumen strains of *Streptococcus bovis* were used. Three strains, 18M/2 (calf), 2SA and 2B (sheep), were kindly supplied by Dr P. N. Hobson of the Rowett Research Institute, Bucksburn, Aberdeenshire. Three other strains, I, 40 and 30, were isolated from fistulated cows feeding on fresh red clover (Bailey & Oxford, 1958*b*).

Two strains of *Leuconostoc mesenteroides*, NCIB 8590 (NRRL, B512) and NCIB 8710 (NRRL, B512F; used for commercial production of dextran) and one strain of *L. dextranicum*, NCIB 8723 were obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex.

Cultural conditions. Streptococcus bovis was grown in a Bacto-Casitone+Bacto Yeast Extract medium (Bailey & Oxford, 1958*a*, *b*) or in a chemically defined medium (Oxford, 1958). Both media contained sucrose (4 %, w/v), glucose or fructose (both 2 %, w/v).

Cultures (1 l.) were incubated at 37° for 48 hr. under 100 % CO_2 gas or in full stoppered flasks from which CO_2 was excluded. Normally the cultures were inoculated from an actively growing 24 hr. culture of the organism in the appropriate sucrose medium. For certain experiments the organisms were serially subcultured six times in glucose medium and the last culture used for inoculation. Only small inocula (4 loopfuls/1 l. medium) were used. The estimated maximum carry-over of sugar in the inoculum was 1–2 mg.

The *Leuconostoc* species were grown in the same Bacto Casitone+Bacto Yeast Extract medium except that it contained sucrose (3 %, w/v), glucose (2 %, w/v), D-arabinose or xylose (both 0·5 %, w/v). The cultures were incubated for 72 hr. at 28° in air. Inoculation conditions were the same as for *Streptococcus bovis* except that inoculation cultures were 48 hr. old and in the case of the pentose cultures inocula (5 ml./1 l. medium) were from 72 hr. cultures in the pentose medium.

Isolation of capsular polysaccharide. Organisms were harvested by centrifuging (15,000 *g*) for 30 min. on a Spinco preparative ultracentrifuge. The bacteria were washed once with acetate buffer (200 ml., 0·05 M, pH 5·0) and then twice with water (200 ml.); in each case by shaking on a wrist-action shaker for 30 min. and re-centrifuging. Capsular polysaccharide was extracted from the organisms by the method of Hobson & Macpherson (1954) with hot sodium acetate+acetic acid solution (pH 2·0). The only modification of the method was that the final ethanol precipitate of deproteinized polysaccharide was dissolved in water (10–20 ml.), centrifuged (30,000 *g*) for 30 min., dialysed for 2 days against distilled water and freeze-dried. The freshly isolated bacteria were examined for capsules by negative nigrosine staining and shown to be always encapsulated. Staining of the organisms after extraction of the capsular material always showed intact almost completely decapsulated organisms (cf. Hobson & Macpherson, 1954).

Hydrolysis of capsular polysaccharides. Polysaccharide (2–5 mg.) in N-HCl (2 ml.) was heated for 6 hr. at 100°. The hydrolysate was evaporated to dryness over solid NaOH in a vacuum desiccator and dissolved in water (0·2 ml.). Polysaccharide (5–10 mg.) was also hydrolysed by incubation with a dextranase preparation (5 mg.) in citrate buffer (0·2 M, pH 5·5, 2 ml.) for 24 hr. at 37°. The final digest was diluted with water, desalted with Dowex 2 and Dowex 50 ion-exchange resins, evaporated to dryness in the desiccator and dissolved in water (0·2 ml.).

Paper chromatographic analysis. Acid hydrolysates were analysed for sugars on paper chromatograms developed with one of the following solvents:

(1) the top layer of a mixture of *n*-butanol, ethanol, water and ammonia sp.gr. 0·88 (49, 10, 40, 1 %, v/v; Barker *et al.* 1955); (2) the top layer of a mixture of ethyl acetate, water and pyridine (2:2:1, v/v; Jermyn & Isherwood, 1949). Paper chromatograms of dextranase hydrolysates were developed with either of these two solvents or with a modification of solvent (2) which was more suitable for separating longer chain oligosaccharides (Malpress & Hytten, 1958).

Sugars, oligosaccharides and uronic acids were detected with silver nitrate (Trevelyan, Procter & Harrison, 1950) and aniline hydrogen phthalate (Partridge, 1949) sprays. Amino sugars were detected with a ninhydrin spray (0·25 % in ethanol).

As it was not possible to carry out quantitative analyses of the sugars in the hydrolysates, a semi-quantitative measure of the amounts of sugars present was obtained by grading the intensity of the sugar spots on the papers in relation to the amount of polysaccharide hydrolysed. A scale scoring from + (trace) to + + + + + + (intense spot) was used. The main concern was whether individual sugars were present in equal, greater or lesser amounts than one another.

Dextranase. A preparation of dextranase (Bailey & Clarke, 1959) was made by precipitation with ammonium sulphate, from cell-free culture fluids of a strain of *Lactobacillus bifidus.* This enzyme hydrolyses both branched and unbranched dextrans to a mixture of *iso*maltotriose, *iso*maltotetraose, *iso*maltopentaose and higher oligosaccharides. The presence, on paper chromatograms of dextranase digests of capsular polysaccharide, of this typical mixture of oligosaccharides was taken as positive evidence for the presence of dextran in the capsular polysaccharide. The enzyme preparation was without action on starch.

RESULTS

Composition of the capsular polysaccharides of the Leuconostoc *species*

The strains of the *Leuconostoc* species examined were grown in media containing various sugars as the sole added carbohydrate. While the organisms grew readily in the media which contained sucrose, glucose or fructose, only poor growth was obtained in the media containing pentose sugars. Both strains of *L. mesenteroides* produced soluble dextrans which remained in solution. The dextran produced in the sucrose culture of *L. dextranicum*, however, was much less soluble and accumulated on the bottom of the culture flask. It was not possible in this culture (L10) to free the organisms entirely from extracellular dextran.

The results obtained from a chromatographic analysis for sugars of acid hydrolysates of the capsular extracts are listed in Table 1. Rhamnose, uronic acids and amino sugars were absent from the papers. None of the capsular extracts gave a stain with iodine indicative of starch or a starch fraction. Portions of capsular extract (Samples L2, L6, L7 and L9) were treated with dextranase and the digests analysed by paper chromatography for *iso*maltodextrins; the results obtained are listed in Table 1. The final dextranase digests

Table 1. *Carbohydrate composition of capsular polysaccharides isolated from* Leuconostoc *species*

All cultures (1 l.) grown for 72 hr. at 28° in Bacto-Casitone + Bacto Yeast Extract medium containing sucrose (3 %, w/v), glucose (2 %, w/v) or pentose (0·5 % w/v).

Sample no.	Sugar substrate	Capsular extract from washed organisms (mg./l. of original culture fluid)	Sugars detected† in hydrolysate of capsular extract			Dextranase test
			Glucose	Galactose	Other sugars	
Leuconostoc mesenteroides, NCIB 8590						
L1	Glucose	13	+ + + +	+ + + +	0	–
L2*	Glucose	32	+ + + +	+ + + +	0	0
L3	Sucrose	15	+ + + +	+ + + +	0	–
L4	Xylose	8	+ + + +	+ + + +	0	–
L5	D-Arabinose	4	+ + + +	+ + + +	0	–
Leuconostoc mesenteroides, NCIB 8710						
L6	Glucose	42	+ + + + + +	+ +	Fructose +	+ + +
L7*	Glucose	28	+ + + + +	+ + +	Fructose +	+
L8	Sucrose	16	+ + + + + +	+ +	.	–
Leuconostoc dextranicum, NCIB 8723						
L9	Glucose	35	+ + + + + +	+	0	+ + +
L10	Sucrose	76	+ + + + + + +	+	0	–

* Inoculated from the last of a series of six successive cultures in glucose medium.

† Scoring of relative intensities of chromatogram spots: + trace to + + + + + + intense.

– Not done.

from samples L6 and L9 were, after dilution with water, boiled for a few minutes, dialysed, centrifuged and concentrated to dryness. These concentrates were hydrolysed in acid and analysed for sugars in the usual manner. Paper chromatograms of the two hydrolysates now showed spots corresponding to approximately equal amounts of glucose and galactose.

Composition of the capsular polysaccharide of Streptococcus bovis

Capsular material was extracted from each strain of *Streptococcus bovis* grown in Casitone yeast extract medium which contained, with one exception, glucose (2 %, w/v) as the sole carbohydrate. The results obtained from chromatographic analyses of acid hydrolysates of the extracts are listed in Table 2. Amino sugars were not detected in any of the hydrolysates. No attempt was made to identify the uronic acid.

Table 2. *Carbohydrate composition of capsular polysaccharide isolated from various strains of* Streptococcus bovis

All organisms were grown for 48 hr. at 37° in Bacto Casitone + Bacto Yeast Extract medium (1 l.) containing glucose (2 %, w/v) except for strain 30 when the medium contained sucrose (4 %, w/v). All cultures were incubated in CO_2 gas (100 %).

Sample no.	Strain no.	Capsular extract from washed organisms (mg./l. of original culture fluid)	Sugars detected* in hydrolysate of capsular extract			
			Glucose	Galactose	Rhamnose	Uronic acid
S1	I	21·0	++++++	+++	++	+
S2	40	3·3	++++++	+++	++	+
S3	30	50·0	+++++++	++	+	0
S4	18M/2	27·0	++++++	+++	++	+
S5	2SA	10·0	+++++	++++	++	0
S6	2B	11·0	++++++	+++	+	+

* Scoring of intensity of chromatogram spots; see Table 1.

Effect of variations in cultural conditions on the composition of the capsular polysaccharide of S. bovis (*strain I*)

Streptococcus bovis (strain I) had been used in a study of the dextransucrase secreted by the organism (Bailey, 1959) and this strain was therefore chosen for this part of the present work. The organism was grown in the Casitone + yeast extract medium and in the chemically defined medium. Both media contained either sucrose, glucose or fructose. Sucrose cultures were grown both in the presence and in the absence of carbon dioxide (Bailey & Oxford, 1958*b*). The results obtained from the analysis of hydrolysates of each capsular extract are listed in Table 3. Amino-sugars were not detected in any of the hydrolysates.

None of the capsular extracts stained with iodine. Portions of capsular material (samples S10, 11, 12 and 13) were tested for dextran by treatment with dextranase and subsequent chromatographic analysis. The results obtained

Table 3. *Effect of various cultural conditions on the composition of the capsular polysaccharide of* Streptococcus bovis (*strain I*)

The organism was grown in medium (1 l.) containing sucrose (4 %, w/v), glucose or fructose (both 2 %, w/v). All cultures were incubated at 37° in CO_2 gas (100%) except where stated otherwise.

Sample no.	Sugar substrate	Capsular extract from washed organisms (mg./l. of original culture fluid)	Sugars detected† in hydrolysate of capsular extract				Dextranase test
			Glucose	Galactose	Rhamnose	Uronic acid	
Bacto-Casitone + Bacto Yeast Extract medium							
S1	Glucose	21	++++++	+++	++	+	–
S7	Fructose	4	++++++	+++	++	+	–
S8	Sucrose	11	++++++	+++	++	+	–
S9	Sucrose (no CO_2)	6	++++++	+++	++	+	–
S10*	Glucose	34	++++	++++	++	+	0
Defined medium							
S11	Sucrose	77	++++++++	+	0	0	+++
S12	Sucrose (no CO_2)	15	+++++++	++	+	+	+++
S13*	Glucose	43	++++	++++	++	+	0

* Inoculated from the last of a series of six successive cultures in glucose medium.
† Scoring as in Tables 1 and 2.
– Not done.

from these tests are listed in Table 3. The final dextranase digests from samples S11 and 12 were freed from *iso*maltodextrins, hydrolysed and analysed by paper chromatography by the methods already described. The two hydrolysates showed on the papers two spots of equal intensity corresponding to glucose and galactose and weaker spots corresponding to rhamnose and uronic acid. A second portion of sample S11 was treated with dextranase and the digest streaked on a paper chromatogram. After development with the modified solvent of Malpress & Hytten (1958) the separated zones of tri- and tetrasaccharides were eluted from the paper with water. Acid hydrolysates of these fractions prepared in the usual manner showed on paper chromatograms a single component corresponding to glucose.

DISCUSSION

In the past it has been tacitly assumed that the type-specific (i.e. capsular) polysaccharide of a dextran-producing bacterium must necessarily contain dextran itself (see Evans & Hibbert, 1946). For rabbit inoculation it is of course usual to grow such bacteria in a glucose (not sucrose) medium with consequent minimal, or no, production of dextran in the culture fluid. The results here recorded indicate, however, that the real capsular polysaccharides, both of the *Leuconostoc* species examined and of *Streptococcus bovis* are not dextran. Dextran may sometimes be present, probably as a contaminant which is not easily removed by simple washing of the organisms at room temperature. Such contamination can occur with organisms grown in sucrose or glucose media. On the other hand, it is possible to obtain organisms, even from sucrose media, which yield a dextran-free capsular polysaccharide.

The results in Table 1 make it clear that the real capsular polysaccharide of true (i.e. dextran-producing) *Leuconostoc* species was not dextran, but a complex containing glucose and galactose in about equal proportions. With *L. mesenteroides* NCIB 8590, it would seem that dextran can all be washed away before extraction of the capsule proper, even with organisms grown on sucrose (see results L1–5, Table 1). With the other two strains of Leuconostoc, however, the dextranase test and the increased ratio of glucose to galactose in the crude capsular extract show that contamination of the true capsular polysaccharide with dextran had occurred even in a glucose medium (see particularly results L6, L7 and L9; Table 1). Result L7 is also noteworthy in that it reveals that the organism produced apparent 'capsular' dextran from glucose even when the inoculum was not only from a glucose medium but from a culture many times removed from a sucrose medium. This raises the interesting question: can the organism actually make sucrose, the only known dextran precursor, from glucose? We have no convincing evidence that this is so. The presence of fructose in the crude capsular polysaccharide of *L. mesenteroides* NCIB 8710 grown in a glucose medium is suggestive (see results L6 and L7, Table 1) as is also the fact that *Streptococcus bovis* produces dextran-sucrase even in a glucose medium (Bailey, 1959; Bailey & Oxford, 1958*a*).

The results obtained with six strains of *Streptococcus bovis* confirm the above conclusions. With this organism the 'real' capsular polysaccharide is more complex than that of Leuconostoc since it contains rhamnose and an uronic acid in addition to galactose and glucose. Results S10 and S13 of Table 3, in particular the dextranase tests, reveal that it was actually possible to grow *S. bovis* in a glucose medium without production of 'capsular' dextran, but only when the inoculum was from a sucrose-free medium. The other results in Tables 2 and 3 show the ease with which 'capsular' dextran can become firmly held as a contaminant of the real capsular polysaccharide even in a glucose medium, when no particular pains are taken to use a sucrose-free inoculum. Bailey & Oxford (1958*a*, *b*) showed that *S. bovis* only produced good yields of extracellular dextran from sucrose in the presence of carbon dioxide. The capsular material isolated from organisms grown on sucrose medium in the absence of carbon dioxide were just as heavily contaminated with dextran as those from the same medium plus carbon dioxide (see results S11 and S12, Table 3). Hobson & Macpherson (1954) found that the capsular polysaccharide of an unidentified rumen streptococcal strain, with all the properties of *S. bovis*, contained only galactose, rhamnose and an uronic acid as constituent sugars. We did not have this strain available. It is possible that our strains all contained two 'real' capsular polysaccharides, one of which was the galactose-rhamnose-uronic acid complex and the other a glucose polymer not identical with dextran or starch. Further work is in hand to clarify this point.

The serological implications of the above chemical results are both obvious and disturbing. It would seem that unless special precautions are taken, or even sometimes when they are taken, confusing cross-reactions between generically unrelated dextran-producing strains may be expected to occur. For a well-known instance from the past involving *Leuconostoc mesenteroides*, *Streptococcus* Group H, and pneumococcal types II and XX, see Dubos (1945) summarizing the work of Neill, Sugg, Hehre & Jaffe (1941). With known soluble dextran-, and perhaps also soluble levan-producing organisms it would seem an obvious precaution not only to use, when possible, media containing neither glucose, sucrose, or fructose but also to ensure that the inoculum used in preparing the culture for conversion to a vaccine is taken from a culture many times removed from a sucrose-containing medium.

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REFERENCES

BAILEY, R. W. (1959). Transglucosidase activity of rumen strains of *Streptococcus bovis*. 2. Isolation and properties of dextransucrase. *Bioohem. J.* (in the Press).

BAILEY, R. W. & CLARKE, R. T. J. (1959). A bacterial dextranase. *Biochem. J.* (in the Press).

BAILEY, R. W. & OXFORD, A. E. (1958*a*). Pre-requisites for dextran production by *Streptococcus bovis*. *Nature, Lond.* **182**, 185.

BAILEY, R. W. & OXFORD, A. E. (1958*b*). A quantitative study of the production of dextran from sucrose by rumen strains of *Streptococcus bovis*. *J. gen. Microbiol.* **19**, 130.

Barker, S. A., Bourne, E. J., James, A. E., Neely, W. B. & Stacey, M. (1955). The structure of a modified *Betacoccus arabinosaceous* dextran. *J. chem. Soc.* p. 2096.

Dubos, R. J. (1945). In *The Bacterial Cell*, p. 121. Camb. Mass. U.S.A.: Harvard University Press.

Evans, T. H. & Hibbert, H. (1946). Bacterial polysaccharides. *Advanc. Carbohyd. Chem.* **2**, 211.

Hobson, P. N. & Macpherson, M. J. (1954). Some serological and chemical studies on materials extracted from an amylolytic Streptococcus from the rumen of the sheep. *Biochem. J.* **57**, 145.

Jermyn, M. A. & Isherwood, F. A. (1949). Improved separation of sugars on the paper partition chromatogram. *Biochem. J.* **44**, 402.

Malpress, F. H. & Hytten, F. E. (1958). The oligosaccharides of human milk. *Biochem. J.* **68**, 708.

Neill, J. M., Sugg, J. Y., Hehre, E. J. & Jaffe, E. (1941). Influence of sucrose upon production of serologically reactive material by certain streptococci. *Proc. Soc. exp. Biol., N.Y.* **47**, 339.

Oxford, A. E. (1958). The nutritional requirements of rumen strains of *Streptococcus bovis* considered in relation to dextran synthesis from sucrose. *J. gen. Microbiol.* **19**, 617.

Partridge, S. M. (1949). Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* **164**, 443.

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, Lond.* **166**, 444.

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FORD, J. E. & GOULDEN, J. D. S. (1959). *J. gen. Microbiol.* 20, 267–276

The Influence of Vitamin B_{12} on Growth Rate and Cell Composition of the Flagellate *Ochromonas malhamensis*

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SUMMARY: In cultures of the flagellate *Ochromonas malhamensis* the rate of growth is determined by the concentration of cyanocobalamin up to a limit at which a maximum growth rate is achieved. In organisms grown exponentially with different rate-limiting concentrations of cyanocobalamin, the amounts of protein and nucleic acids were approximately proportional to the rates at which the organisms had grown. With increasing growth rate the concentrations of riboflavin, nicotinic acid, pantothenic acid and chlorophylls increased. Carbohydrate showed little change, whereas fat and 'free' amino-nitrogen diminished in concentration as the growth rate increased. In cultures of *O. malhamensis* grown with limiting or near-limiting concentrations of cyanocobalamin and with increasing concentrations of chloramphenicol, the rates of division declined asymptotically to a low value corresponding with 500 μg. chloramphenicol/ml. culture medium. Inhibition of growth caused by the antibiotic was annulled by increasing the concentration of cyanocobalamin in the culture medium.

A variety of cultural and nutritional factors can influence the balance of composition of living cells. Vitamin deficiencies, for example, may distort the cell metabolism and bring about characteristic 'biochemical lesions'. Vitamin B_{12} plays presumably a part in diverse metabolic processes, but in some of these its action is probably indirect. No single fundamental role can as yet be assigned to this vitamin, although there are indications, mainly from work with animals and animal-tissue preparations, that its essential function is concerned with protein biosynthesis (see review by Smith, 1958). If indeed the vitamin acts in protein or nucleic acid synthesis, this action would give rise to a variety of secondary effects and explain an apparent multiplicity of functions. For example, it would explain the low concentrations of certain enzymes found in the tissues of deficient animals (Mistry *et al.* 1955; Wong & Schweigert, 1957; Murthy, Desikachar & Swaminathan, 1956); the sharp decrease in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in vitamin B_{12}-deficient *Euglena gracilis* (Soldo, 1955) and of DNA in *Lactobacillus leichmannii* (Wacker, Pfahl & Schröder, 1957); and the effect of vitamin B_{12} in reversing the actions of aminopterin and aureomycin on the synthesis of DNA in *L. leichmannii* (Rege & Sreenivasan, 1954*a*, *b*).

In several species of bacteria the nucleic acid content of the organisms is related to the rate at which they have grown (Malmgren & Hedén, 1947). In *Bacterium lactis aerogenes* the growth rate under a variety of cultural conditions is indeed proportional to the cell content of RNA (Caldwell, Mackor & Hinshelwood, 1950). In cultures of the flagellate *Ochromonas malhamensis* the rate of growth is determined by the concentration of vitamin B_{12} up to a limit at which a maximum growth rate is achieved. This relationship is described by an equation of the form of an adsorption isotherm (Ford, 1958). The present paper is concerned with the concentrations of proteins, nucleic acids, fat, carbohydrate and of vitamins representative of major enzyme systems, in *O. malhamensis* grown with different limiting concentrations of vitamin B_{12} and harvested within the period of exponential growth. An effect of vitamin B_{12} in reversing inhibition of growth caused by chloramphenicol is also reported.

METHODS

Growth of organisms and preparation for analysis

The composition of the culture media, and the general methods used for maintaining the organism and for preparing inocula, were as described previously (Ford, 1953, 1958). Cultures used to provide the organisms for analysis were incubated in darkness at 30° in vitamin B_{12}-free basal medium supplemented with 0·05, 0·10 0·20 or 4·0 mμg. cyanocobalamin/ml. At each of these concentrations ten replicate cultures were grown, in 100 ml. portions of medium contained in 10 oz. 'medicine flats'. The bottles of medium were plugged with cotton wool and autoclaved for 10 min. at 115°. After cooling to room temperature each was then inoculated with 1 ml. of a 4-day culture (cf. Ford, 1958), placed in a shaking machine in an air incubator operating at 29°, and incubated for 46 hr. After this relatively short period of incubation the yield of organisms was only about one-third of the maximum obtainable, but growth was strictly exponential only during these early hours of incubation, and after 48 hr. the rate of division began to decline markedly in the cultures grown with the higher concentrations of vitamin B_{12}. On being removed from the incubator the replicate cultures were combined and their optical densities at 580 mμ determined in a Lumetron model 400 A photometer. At this stage there were four bulked cultures, each of them comprised of ten replicate 100 ml. portions. These bulked cultures were now centrifuged separately for 20 min. at 1500 g. The supernatant fluid was drained as completely as possible from the deposited organisms, which were resuspended in 2–3 ml. of water, frozen quickly and freeze-dried. The dried organisms were ground finely and stored in a desiccator over P_2O_5.

Ochromonas malhamensis is facultatively photosynthetic, and for one experiment the organism was grown in light, at different concentrations of cyanocobalamin in a culture medium containing NH_4Cl (0·3 g./100 ml.) as sole nitrogen source. Cultures were grown in 400 ml. portions of medium in 'penicillin bottles' at 27° and without shaking under uniform illumination at 180 foot-candles. Under these conditions growth was slower and a longer incubation period (9 days) was used. The organisms were otherwise prepared for analysis as described above.

Infra-red absorption spectra. The infra-red absorption spectrum of each of the dried cell preparations was examined by the method described by Goulden & Sharpe (1958).

Determination of cell constituents

Nitrogen. Total cell nitrogen was determined by micro-Kjeldahl analysis. 'Free' amino-nitrogen was extracted from the cells with cold 10% (v/v) aqueous acetic acid (cf. Mattick *et al.* 1956) and estimated with ninhydrin reagent by the method of Yemm & Cocking (1955).

Nucleic acids. Total nucleic acid was measured by ultraviolet absorption at 268 mμ, and deoxyribonucleic acid by reaction with indole, with a purified preparation of calf thymus DNA as reference standard; ribonucleic acid was calculated by difference. The procedures followed were those of Ceriotti (1955) as modified by Paul (1958).

Carbohydrates. Total carbohydrate was determined by the anthrone method, as described by Trevelyan & Harrison (1952).

Fat. Fat was extracted from the dried organisms with diethyl ether, and was estimated by difference.

Vitamins of the B-complex. Pantothenic acid, nicotinic acid, riboflavin and the vitamin B_6 group were determined by microbiological assay. The methods used were as described by Gregory, Ford & Kon (1958).

Chlorophylls. Chlorophylls were determined spectrophotometrically by the method described by Richards (1952).

RESULTS

The values obtained for nitrogen, nucleic acids, carbohydrate and fat are set out in Table 1. With increasing concentration of cyanocobalamin the rate of growth of the organism increased, as did the content of nitrogen, RNA and DNA. Total carbohydrate showed little or no change; fat and 'free' amino-nitrogen diminished.

The relation between growth rate and cyanocobalamin concentrations in the culture medium is described by the equation, $k/k_{\max} = C/(C_1+C)$, in which k is the relative growth rate and $k_{\max}$ the rate when $C = \infty$; C is the

Table 1. *Growth rate and composition of* Ochromonas malhamensis *grown at different concentrations of cyanocobalamin*

Conditions of cultivation	Cyanocobalamin in culture medium (mμg./ml.)	Growth rate (k)*	Percentage organism dry wt.					
			Total nitrogen	'Free' amino-nitrogen	RNA	DNA	Carbohydrate	Fat
Grown in darkness in complete medium	0·05	2·55	2·83	0·30	1·61	0·95	52·0	7·0
	0·10	2·85	3·40	0·26	1·92	1·00	47·5	3·6
	0·20	3·02	3·90	0·23	2·13	1·02	52·0	1·3
	4·0	3·20	4·06	0·24	2·26	1·21	52·0	0
Grown in light in minimal medium	0·06	—	4·96	0·50	3·20	0·82	42·0	20·0
	0·60	—	5·60	0·35	3·72	0·82	40·5	15·6

* No. divisions/24 hr.

concentration of cyanocobalamin, and C_1 is the value of C at which $k = \frac{1}{2}k_{\max}$ (Ford, 1958). Some further possible relationships between growth rate in the 'complete' medium and cell composition are indicated in Table 2. These results indicate a close relation between the rate at which the organisms had grown and their content of nucleic acids and total nitrogen. A similar close proportionality was evident in the cell concentrations of nitrogen and nucleic acid.

Table 2. *Some correlations between growth rate* (k), *nitrogen and nucleic acids in* Ochromonas malhamensis *grown with different concentrations of cyanocobalamin*

Cyanocobalamin in culture medium (mμg./ml.)	$\frac{\text{Nucleic acids}}{k}$	$\frac{\text{Nitrogen}}{k}$	$\frac{\text{Nitrogen}}{\text{Nucleic acids}}$
0·05	1·00	1·10	1·10
0·10	1·02	1·19	1·16
0·20	1·04	1·29	1·24
4·0	1·08	1·27	1·17

It had been considered inadvisable to wash the organisms before drying and the deposited organisms obtained by centrifuging the cultures were simply drained of supernatant fluid as completely as possible. The residual entrained culture fluid might have introduced a systematic error into the figures representing 'free' amino-nitrogen in the organisms grown in complete medium. However, the values for 'free' amino-nitrogen in the organisms grown in light in minimal medium (NH_4Cl as nitrogen source) showed the same reciprocal relationship to growth rate and are not suspect for this reason.

There were clear differences in composition between organisms grown in darkness in complete medium and those grown in light in minimal medium. These latter were much higher in fat; the concentrations of nitrogen, RNA and B_2-complex vitamins (see p. 272), were also comparatively high, whereas DNA and carbohydrate were lower. No increase in DNA concentration with increase in cyanocobalamin was observed, but in other respects the influence of the vitamin on cell composition was the same under both sets of cultural conditions. No estimates of growth rate were made because growth of the organism in minimal medium was relatively slow, and was probably not strictly exponential throughout the incubation period of 211 hr. used.

Tween 80 (polyoxyethylene sorbitol mono-oleate) stimulates the growth of *Ochromonas malhamensis* and was included in both minimal and complete culture media. It was observed that in the presence of this compound the requirement for biotin reported by Hutner, Provasoli & Filfus (1953) could not be demonstrated. The accumulation of fat in the vitamin B_{12}-deficient cultures was not related to the presence of Tween 80. There was apparently a reciprocal relation between growth rate and fat content, and, in fact, the omission of Tween 80 from the culture media caused a significant increase in the concentration of cell fat, together with a slight retardation of growth.

Infra-red absorption spectra. The relation between vitamin B_{12} content and the fat and protein content of *Ochromonas malhamensis* is illustrated in a comparison of the infra-red absorption spectra of organisms grown at different concentrations of vitamin. Figure 1 shows the absorption, in the 2000 to 830 cm.$^{-1}$ region, of organisms grown in minimal medium in light (curves A and B), and of organisms grown in complete medium in darkness (curves C and D), with limiting amounts or excess of cyanocobalamin. The vitamin B_{12}-deficient organisms (curves B and D) showed higher absorption at 1730 cm.$^{-1}$ and lower absorption in the 1650 and 1540 cm.$^{-1}$ bands. The absorption at 1730 cm.$^{-1}$ disappeared on extraction of the organisms with diethyl ether, and the spectrum of the extracted material suggested that it consisted largely of saturated triglyceride fats. The absorption at 1650 and 1540 cm.$^{-1}$ can be attributed in the main to the cell proteins (Rideal & Adams, 1957), and was of lower intensity in the organisms grown with limiting amounts of cyanocobalamin. The strong absorption bands in the 1050 cm.$^{-1}$ region were due mainly to polysaccharides (Rideal & Adams, 1957) and were of equal intensity in the organisms grown with limiting amounts or with excess of cyanocobalamin.

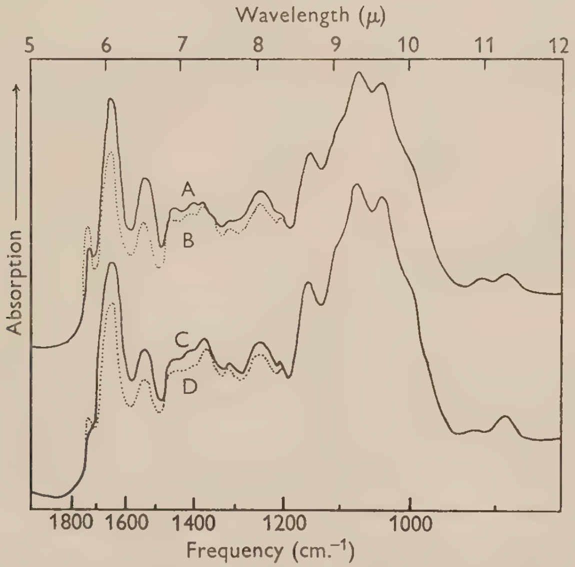

Fig. 1. Infra-red absorption spectra of dried *Ochromonas malhamensis*. Curves A, B: organisms grown in minimal medium in light, with limiting (B) or with excess (A) cyanocobalamin. Curves C, D: organisms grown in complete medium in darkness, with limiting (D) or with excess (C) cyanocobalamin.

Vitamin content of organisms. The preparations of organisms grown in 'complete' medium (see Table 1) were analysed for riboflavin, nicotinic acid, pantothenic acid and the vitamin B_6 group. The values obtained are given in Table 3; for comparison, values are also given for organisms grown in light in minimal medium. The acceleration of growth brought about by cyanocobalamin was matched by increased cell concentrations of nicotinic acid, riboflavin and pantothenic acid. 'Vitamin B_6', on the other hand, was lowest in organisms grown with cyanocobalamin in excess.

Table 3. *Vitamin content of* Ochromonas malhamensis *grown at different concentrations of cyanocobalamin*

Conditions of cultivation	Vitamin concentration (μg./g. dried cells) Nicotinic acid	Riboflavin	Pantothenic acid	'Vitamin B_6'
Complete medium* + cyanocobalamin (mμg./ml.)				
0·05	49	12	3·0	4·6
0·10	53	14	4·3	5·0
0·20	54	16	5·5	4·6
4·0	62	18	6·0	2·9
Minimal medium + cyanocobalamin (mμg./ml.)				
0·025	59	20	5·0	5·8
0·20	64	28	6·2	2·8

* Same organisms as described in Table 1.

Distribution of vitamins between organisms and culture fluid. Nicotinic acid, pantothenic acid and the vitamin B_6 group were measured in the organisms and culture fluids of a culture grown in complete medium. These three vitamins were initially absent from the medium, and the sum of the vitamin content of the organisms and that of the culture fluid in which they had grown represents net synthesis. The results showed that even during the period of exponential growth, loss of these vitamins from the organisms to the surrounding medium constituted the main burden on synthesis. The ratio, amounts of these vitamins in the organisms/ml. culture: amounts/ml. culture fluid, was about 1:6 for pantothenic acid, about 1:3 for nicotinic acid and about 1:15 for the vitamin B_6 group. These figures refer to a culture grown for 42 hr. with limiting amount of cyanocobalamin (0·2 mμg./ml.).

Chlorophylls. When grown in light in minimal medium, the concentration of chlorophylls in cyanocobalamin-deficient organisms was lower than in controls grown with the vitamin in excess of the requirement for maximum growth rate. The chlorophyll contents of the organisms grown in light with 0·06 and 0·60 mμg. cyanocobalamin/ml. were 1·80 and 2·85 μg./mg. dried organism, respectively.

Glutathione. Attempts to measure glutathione by reaction with nitroprusside (Grunert & Phillips, 1951) were unsuccessful. This method of assay was not sufficiently sensitive to reveal the presence of glutathione in the cell preparations, either before or after electrolytic reduction. It seems safe to presume that the dried organisms contained less than 0·2 μg. glutathione/mg. dry wt.

Tests with chloramphenicol. Gale & Folkes (1953) showed that with *Staphylococcus aureus* chloramphenicol at suitable concentration acted selectively in preventing protein synthesis; other cell processes, including nucleic acid synthesis and the accumulation of free amino acids, were not inhibited except at very high concentrations of the antibiotic. In cultures of *Ochromonas malhamensis* grown with limiting amounts of cyanocobalamin and with increasing concentrations of chloramphenicol, the rates of division declined asymptotically to a low value at 500 μg. chloramphenicol/ml. culture medium. It seemed that the relation between growth rate and chloramphenicol concentration might be the inverse of that linking growth rate with cyanocobalamin concentration, but this possibility was not investigated in detail.

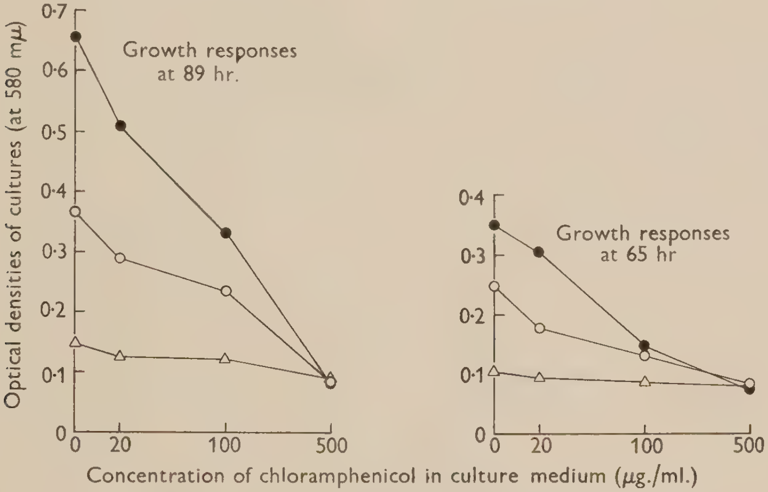

Fig. 2. Influence of chloramphenicol on the growth of *Ochromonas malhamensis* in media containing cyanocobalamin at: (1) 0·03 mμg./ml. (△—△); (2) 0·1 mμg./ml. (○—○); (3) 0·3 mμg./ml. (●—●).

Evidence that vitamin B_{12} and chloramphenicol may be concerned in the same metabolic processes can be adduced from the finding (Fig. 2) that inhibition of growth caused by chloramphenicol was annulled by increasing the concentration of cyanocobalamin. This effect might, of course, be indirect. Miyamura (1958) showed with *Escherichia coli* an annulment of a growth-inhibitory effect of chloramphenicol by certain amino acids and peptides.

Effects of chloramphenicol on the composition of organisms. Analyses were made of dried *Ochromonas malhamensis* grown in complete medium with excess cyanocobalamin, with and without chloramphenicol (50 μg./ml.). Growth in the presence of chloramphenicol was slow and the organisms were relatively rich in fat (as judged by the absorption at 1730 cm.$^{-1}$), and in protein, RNA and DNA, all of which were about 12 % higher than in the control organisms. The carbohydrate content was markedly lower, being 44 % of the dry weight of organisms as compared with 58 % in the control organisms. 'Free' amino-nitrogen showed an increase of *c.* 5 %. Thus chloramphenicol and vitamin B_{12}-depletion had clearly different effects on the protein and nucleic acid composition of the organisms.

DISCUSSION

Vitamin B_{12} depletion in *Ochromonas malhamensis* brings about changes in composition that have been recognized in various algal species as being highly characteristic of nitrogen deficiency (Collyer & Fogg, 1955; Spoehr & Milner, 1949; for a review see Fogg, 1959). This finding suggests that in *O. malhamensis* the vitamin is concerned with the elaboration of nitrogen compounds; the vitamin B_{12}-deficient organisms may be in a sense deficient in nitrogen. Wagle, Mehta & Johnson (1958) believe that vitamin B_{12} functions essentially in the incorporation of amino acids into proteins; they reported a striking effect on this incorporation in preparations of tissues from vitamin B_{12}-deficient rats. Other workers (Fraser & Holdsworth, 1959; Arnstein & Simkin, 1959) were unable to confirm these findings, and suggested that the decreased protein synthesis in vitamin B_{12} deficiency might be secondary to some other function of the vitamin. Apart from these equivocal findings of Wagle *et al.* (1958), most of the evidence which links vitamin B_{12} with protein biosynthesis is circumstantial. However, the syndrome of vitamin B_{12} deficiency in *O. malhamensis* seems altogether compatible with an over-riding function of the vitamin in protein biosynthesis, and the relationships between cyanocobalamin concentration, growth rate and cell composition suggest that a re-orientation of thinking around this problem may be helpful. To seek to demonstrate a dramatic potentiation of enzyme function upon the addition of cyanocobalamin to deficient systems might be to seek the proverbial mare's nest. Reference to Table 1 shows that in *O. malhamensis* the effect of severe deprivation of vitamin B_{12} was to decrease the rate of cell division by only 20 %. The rate of protein biosynthesis, and so presumably of amino acid incorporation, was decreased by a slightly greater amount. If we consider two populations of *O. malhamensis* growing exponentially, a difference of 20 % in their relative growth rates would quickly lead to a wide divergence between their relative population densities. If this small difference in rates of amino acid incorporation were the essential difference between the normal and the vitamin B_{12}-deficient organism, the effect on growth would probably be a summation of small effects on many enzymes, and might not be demonstrable as an all-or-nothing effect on any one system.

Ochromonas malhamensis grown at low concentrations of cyanocobalamin was relatively low in protein and nucleic acids, whereas organisms whose growth had been inhibited by chloramphenicol were over-full of both these constituents. This observation argues against the view that chloramphenicol and vitamin B_{12} exert their opposite effects on growth at the same site in protein synthesis. It seems to rule out the possibility that chloramphenicol blocks the action of the vitamin by saturating the vitamin B_{12}-binding mechanism (Ford, 1958).

The authors wish to express their gratitude to Dr S. K. Kon for his wise counsel and helpful criticism. They are indebted also to Dr L. A. Mabbitt for the measurements of 'free' amino-nitrogen, and to Dr J. A. F. Rook for the total-N determinations.

REFERENCES

ARNSTEIN, H. R. V. & SIMKIN, J. L. (1959). Influence of vitamin B_{12} on protein biosynthesis in rat liver. *Nature, Lond.* (in the Press).

CALDWELL, P. C., MACKOR, E. L. & HINSHELWOOD, C. (1950). The ribose nucleic acid content and cell growth of *Bact. lactis* aerogenes. *J. chem. Soc.* p. 3151.

CERIOTTI, G. (1955). Determination of nucleic acids in animal tissues. *J. biol. Chem.* **214**, 59.

COLLYER, D. M. & FOGG, G. E. (1955). Studies on fat accumulation by algae. *J. expt. Bot.* **6**, 256.

FOGG, G. E. (1959). Nitrogen nutrition and metabolic patterns in algae. *Symp. Soc. exp. Biol.* (in the Press).

FORD, J. E. (1953). The specificity of the requirement of *Ochromonas malhamensis* for cyanocobalamin. *Brit. J. Nutrit.* **7**, 299.

FORD, J. E. (1958). B_{12}-vitamins and growth of the flagellate *Ochromonas malhamensis*. *J. Gen. Microbiol.* **19**, 161.

FRASER, M. J. & HOLDSWORTH, E. S. (1959). Investigation of possible function of vitamin B_{12} in protein biosynthesis in chick liver. *Nature, Lond.* (in the Press).

GALE, E. F. & FOLKES, J. P. (1953). The assimilation of amino acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochem. J.* **53**, 493.

GOULDEN, J. D. S. & SHARPE, M. E. (1958). The infra-red absorption spectra of lactobacilli. *J. gen. Microbiol.* **19**, 76.

GREGORY, M. E., FORD, J. E. & KON, S. K. (1958). The B-vitamin content of milk in relation to breed of cow and stage of lactation. *J. Dairy Res.* **25**, 447.

GRUNERT, R. R. & PHILLIPS, P. N. (1951). A modification of the nitroprusside method of analysis for glutathione. *Arch. Biochem.* **30**, 217.

HUTNER, S. H., PROVASOLI, L. & FILFUS, J. (1953). The nutrition of freshwater chrysomonads. *Ann. N.Y. Acad. Sci.* **55**, 852.

MALMGREN, B. & HEDÉN, C. (1947). Studies of the nucleotide metabolism of bacteria. III. The nucleotide metabolism of the Gram negative bacteria. *Acta path. microbiol. scand.* **24**, 448.

MATTICK, A. T. R., CHEESEMAN, G. G., BERRIDGE, N. J. & BOTTAZZI, U. (1956). The differentiation of lactobacilli and streptococci by means of paper partition chromatography. *J. appl. Bact.* **19**, 310.

MISTRY, S. P., VADOPALAITE, I., CHANG, I., FIRTH, J. & JOHNSON, B. C. (1955). Vitamin B_{12} and transmethylation in pig, chick and rat liver homogenates, *J. biol. Chem.* **212**, 713.

MIYAMURA, S. (1958). Bactericidal action of chloramphenicol on *Escherichia coli*. *J. Bact.* **75**, 617.

MURTHY, V. S., DESIKACHAR, H. S. R. & SWAMINATHAN, M. (1956). Effect of vitamin B_{12} deficiency on liver dehydrogenase activity in rats. *Nature, Lond.* **177**, 750.

PAUL, J. (1958). Determination of the major constituents of small amounts of tissue. *Analyst*, **83**, 37.

REGE, D. V. & SREENIVASAN, A. (1954*a*). The influence of folic acid and vitamin B_{12} on nucleic acid metabolism in microorganisms. *J. biol. Chem.* **210**, 373.

REGE, D. V. & SREENIVASAN, A. (1954*b*). Influence of folic acid and vitamin B_{12} on the impairment of nucleic acid synthesis in *Lactobacillus casei* by aureomycin. *Nature, Lond.* **173**, 729.

RICHARDS, F. A. (1952). The estimation and characterization of plankton populations by pigment analyses. *J. mar. Res.* **11**, 147.

RIDEAL, E. K. & ADAMS, D. M. (1957). The interpretation and use of bacterial infrared spectra. *Chem. & Ind.* p. 762.

SMITH, E. L. (1958). The biochemical functioning of vitamin B_{12}. *Nature, Lond.* **181**, 305.

SOLDO, A. T. (1955). Vitamin B_{12} and nucleic acid production in *Euglena*. *Arch. Biochem. Biophys.* **55**, 71.

SPOEHR, H. A. & MILNER, H. W. (1949). The chemical composition of chlorella; effect of environmental conditions. *Plant Physiol.* **24**, 120.

TREVELYAN, W. E. & HARRISON, J. S. (1952). Studies on yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *Biochem. J.* **50**, 298.

WACKER, A., PFAHL, D. & SCHRÖDER, I. (1957). Beziehungen zwischen Vitamin B_{12} und der Desoxyribonucleinsäure. *Z. Naturf.* **12**b, 510.

WAGLE, S. R., MEHTA, R. & JOHNSON, B. C. (1958). Vitamin B_{12} and protein biosynthesis. *J. biol. Chem.* **233**, 619.

WONG, W. T. & SCHWEIGERT, B. S. (1957). Role of vitamin B_{12} in nucleic acid metabolism. Liver deoxyriboaldolase activity. *Proc. Soc. exp. Biol., N.Y.*, **94**, 455.

YEMM, E. W. & COCKING, E. C. (1955). The determination of amino acids with ninhydrin. *Analyst*, **80**, 209.

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Hill, L. R. (1959). *J. gen. Microbiol.* **20**, 277–283

The Adansonian Classification of the Staphylococci

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SUMMARY: The Adansonian method of classification, in which all features are given equal weight in deducing similarity between strains, has been applied to staphylococci. *Staphylococcus aureus*, *S. saprophyticus* and *S. roseus* are natural groups, but *S. lactis* and *S. afermentans* are not, for individual strains of these 'species' are as different from each other as from the three previous species. The group as a whole is divided into two main branches which might be regarded conveniently as two genera, *Staphylococcus* and *Micrococcus*.

A new approach to bacterial systematics was proposed by Sneath (1957*a*, *b*) in which taxonomic features were handled mathematically to calculate similarity (S) values between strains. Strains were then sorted into groups of like S values. This system is different from classical taxonomy, not so much in its novel mechanics, as in a shift away from the view that some features of an organism are more important than others for classification purposes. Systematic bacteriology appears to be more an art than a science, the art of choosing important features. We know from experience with higher organisms that features which are conveniently regarded as important are essentially correlated features. In the 'Adansonian' approach to taxonomy, advocated by Sneath, bias in choosing important features is eliminated by giving all features equal weight initially. A wide range of features is taken, a percentage similarity between strains calculated, and groups are deduced impartially. Correlated features should then arise as the result of classification and not as a pre-requisite to it. Sneath's original papers should be consulted for the theoretical and practical aspects of this method of taxonomy.

The staphylococcus-micrococcus group of organisms has always been an enigma to bacterial systematists. The working classification of the National Collection of Type Cultures (NCTC), proposed by Shaw, Stitt & Cowan (1951), consists of five species only. In *Bergey's Manual* (1957) there are forty-three species in the family Micrococcaceae. The group has led to at least one unorthodox classification by Abd-el-Malek & Gibson (1948), who regarded it as forming a linear series, at one end of which were the pathogenic staphylococci and, at the other end, a thermoduric saprophyte (red micrococci were omitted). Although they believed that the series was essentially continuous, 'nodal points' (Wilson, 1955) were distinguishable at which numerous strains were grouped together. These could have been given Linnaean-like names, though in fact this was not done.

It was thought that (*a*) the Adansonian method might provide, at last, a good classification for the group, and (*b*) that staphylococci should form a rigorous proving-ground for a method of taxonomy which seems to have great possibilities.

METHODS

Strains were collected from nasal swabs of healthy persons, purulent lesions, animal carcasses, milk, air, the NCTC, and the collection of the Botany Department of the University College, Cardiff. They were described in terms of eighty features which are listed in Table 1.

Table 1. *List of features used as differential characters in this study*

Test	No. of features	Test	No. of features
Gram reaction	2	Fermentation of	
Diameter of cells	4	Glucose	1
Cell arrangements	6	D(+)-xylose	1
Colonial morphology	3	Lactose	1
Degree of golden pigmentation	3	Sucrose	1
Pink, yellow and white pigments	1 each	Maltose	1
		Mannitol	1
Optimal temperature	2	Salicin	1
Growth in nutrient broth	2	Voges-Proskauer	1
Pellicle	1	Methyl red	1
Degree of anaerobic growth	2	Urease	2
Catalase	1	Degree of gelatin liquefaction	2
Bound coagulase	2	Nitrate reduction	1
Free coagulase	3	Methylene blue reduction	2
α-Haemolysin	3	NH_4 salt utilization	2
β-, δ-, ε-Haemolysins	1 each	Caseinase	2
		Degree of serum digestion	2
Lipase	1	NaCl tolerance	3
Phosphatase	1	Heat sensitivity	3
Fibrinolysin	2	Phenol sensitivity	4
Flocculation lines	2		
Penicillinase	2		

Morphology

Two degrees of Gram-positiveness were recognized, dependent upon the ease of decolorization. Three groups were delineated by measuring cell diameters, 0·6–0·8, 0·9–1·0 and 1·1–1·3 μ. Cell arrangements recognized were 'bunches of grapes', loose-lying, tetrads, cubes of eight, chains of more than five cells, and the presence of pleomorphic cells (individual cells considerably larger than the majority in the smear).

Cultures grown on nutrient agar at 37° for 48 hr. were characterized as minute streptococcal-like colonies, typical staphylococcal colonies either 'Rough' or 'Smooth', and having tackiness determined with a wire loop.

From cultures grown on 30 % milk agar at 37° for 48 hr. followed by 3 days on the bench, pigments recognized were golden yellow (1+, 2+ or 3+), pink, lemon-yellow, and white.

Physiology

Nutrient agar cultures were grown at 22°, 30° and 37° to determine optimal growth temperature. Growth in nutrient broth (after 24 hr. at 37°) was described as uniform turbidity with compact deposit, or slight turbidity with granular diffuse deposit. Surface ring pellicles were also noted.

Growth under anaerobic conditions (McIntosh and Fildes's jar) was described as profuse or reduced.

Loopfuls of test organisms were emulsified into drops of 20 vol. H_2O_2 on a slide and recorded as catalase positive when bubbles were evolved.

'Bound' coagulase (slide test, Duthie, 1954) was described as causing rapid or slow clumping, and 'free' coagulase (tube test) divided into 1+, 2+ or 3+ categories according to titre.

Elek & Levy's (1950*b*) nomenclature for the haemolysins was followed as their technique was used. The α-haemolysin was described as 1+, 2+ or 3+ according to the measured width of the zone of lysis, and also recognized were the β-, δ- and ϵ-haemolysins.

Lipase was detected on Marks's (1952) tributyrin agar and phosphatase by phenolphthalein phosphate agar (Barber & Kuper, 1951).

Fibrinolysin (1+ or 2+) was detected with precipitated fibrin agar plates (Lack, 1957).

Flocculation lines produced against staphylococcal antitoxin paper strips (Elek & Levy, 1950*a*) were also divided into 1+ and 2+ (1–4 lines, and more than four lines produced, respectively).

Strains were divided into 1+ and 2+ producers of penicillinase by using the method of Parker & Lapage (1957) in which the Oxford staphylococcus was used as a standard.

Biochemistry

Nutrient agars incorporating various carbohydrates and bromcresol purple indicator were used for identifying fermentation reactions. (This method is probably less sensitive than a tube method, but it serves most efficiently to separate two main groups ultimately found.)

Voges-Proskauer (Barritt's modification) and methyl red reactions were carried out as described by Mackie & McCartney (1953).

Urease was detected on Christensen's urea agar slants incubated at 37° and described as 1+ or 2+.

Nutrient gelatin stab cultures were incubated at 22° for up to 30 days and liquefaction was also described as 1+ or 2+.

Sulphanilic acid and α-naphthylamine reagents were used in the nitrate reduction to nitrite test on 5-day cultures grown in nitrate broth at 37°.

Decolorization of methylene blue added to nutrient broth cultures was described as partial or complete.

Hucker's (*Bergey's Manual*, 6th ed., 1948) medium was used to detect ammonium salt utilization and results were recorded as growth only, or growth with acid production.

20% (v/v) milk agar gave clear indication of casein hydrolysis.

Serum digestion on Loeffler's slopes incubated at 37° was described as 1+ or 2+.

Sodium chloride tolerance was determined with 9, 12 and 15% (w/v) NaCl nutrient agars; heat and phenol sensitivities by the methods of Sneath (1956).

Both the method of deriving Adansonian classifications and the systems of scoring quantitative data are described by Sneath (1957*b*).

RESULTS

The organisms were provisionally classified according to the scheme of Shaw *et al.* (1951). They comprised: 20 strains of *Staphylococcus aureus*; 8 strains of *S. saprophyticus*; 9 strains of *S. lactis*; 7 strains of *S. roseus*; 5 strains of *S. afermentans*.

An $i \times m$ (strains × features) table was drawn up, % S values for each pair of strains were calculated, and an $i \times i$ (strains × strains) table was prepared. Groups were sorted from these % S values and intra- and inter-group mean square S values further calculated from a rearranged $i \times i$ table (which is represented in Fig. 1). A taxonomic tree was then designed and is presented here as Fig. 2. Two main branches were found which may tentatively be regarded as two genera.

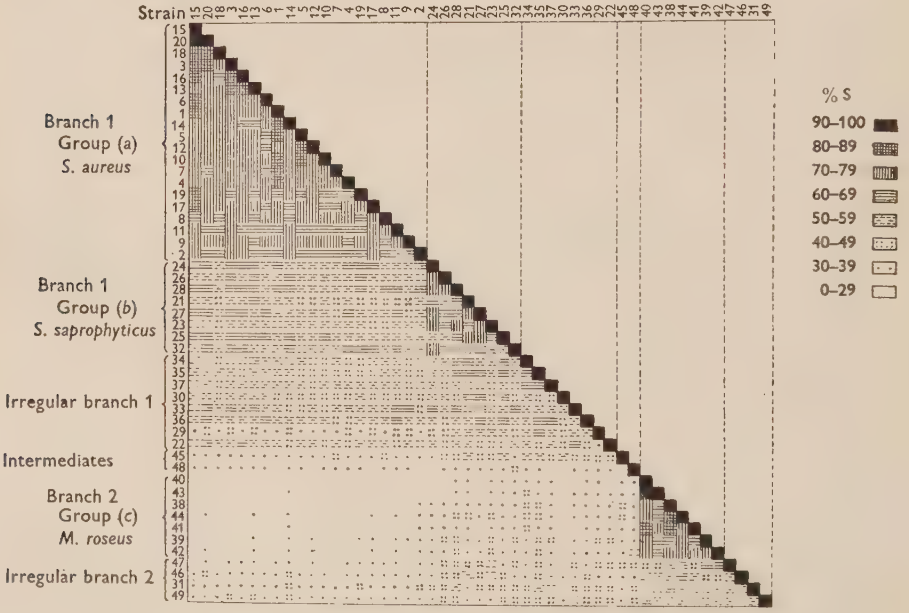


Fig. 1. Diagrammatic representation of the full % S value table.

Three of the groups defined by the Shaw *et al.* scheme remained as groups when subjected to Adansonian analysis. These were (*a*) *Staphylococcus aureus*, (*b*) *S. saprophyticus*, and (*c*) *S. roseus*. However, from the diagram it can be seen that groups (*a*) and (*b*) join together at a higher % S level than they do with group (*c*), indicating that *S. aureus* and *S. saprophyticus* are more closely related than they are to *S. roseus*. Also, single strains joined independently on to one or other of the two main branches, all at much the same % S level. Two strains were intermediate between the two main branches.

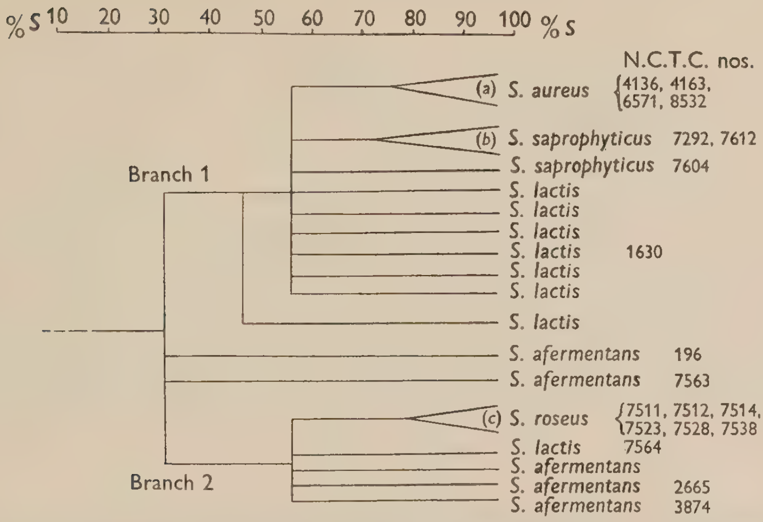

%S 10 20 30 40 50 60 70 80 90 100 %S
N.C.T.C. nos.
(a) S. aureus {4136, 4163, 6571, 8532
(b) S. saprophyticus 7292, 7612
S. saprophyticus 7604
Branch 1
S. lactis
S. lactis
S. lactis
S. lactis 1630
S. lactis
S. lactis
S. lactis
S. afermentans 196
S. afermentans 7563
(c) S. roseus {7511, 7512, 7514, 7523, 7528, 7538
S. lactis 7564
S. afermentans
Branch 2
S. afermentans 2665
S. afermentans 3874

Fig. 2. Taxonomic tree of the group according to Adansonian analysis. (Names are those according to the Shaw *et al.* 1951 scheme.)

DISCUSSION

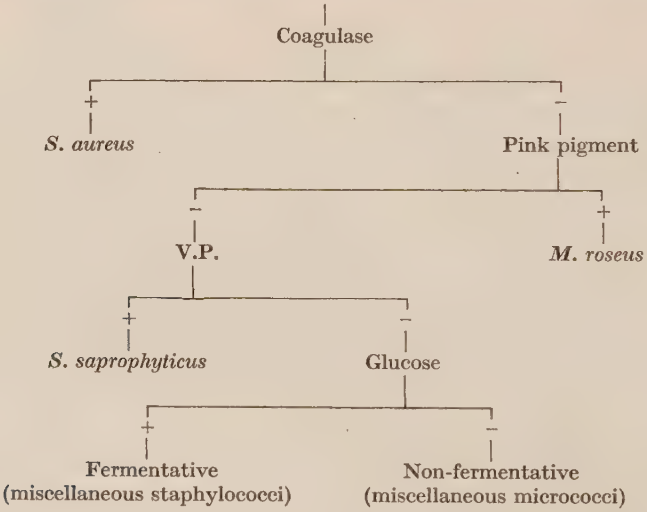
The three species *Staphylococcus aureus*, *S. saprophyticus* and *S. roseus* are natural groups and can be accepted as logical classification. The possession of the same generic name, however, implies that these three species are equally closely related to one another. The Adansonian classification, on the other hand, clearly indicates that *S. aureus* and *S. saprophyticus* are much more closely related to each other than they are to *S. roseus*. A truer reflexion of the relationships of these three species would be shown if the name *Staphylococcus roseus* was changed to *Micrococcus roseus*. The generic term *Staphylococcus* could then be reserved for branch 1 in the Adansonian classification, comprising strains typically from human or animal sources and capable of fermenting a wide range of sugars (by the plate technique). *Micrococcus* could be used for strains of branch 2, which are not typically parasitic and which are generally inactive biochemically, especially in lacking fermentative powers (by the same technique).

If branches 1 and 2 of the Adansonian classification are to be regarded as two genera, *Staphylococcus* is undoubtedly the correct name for branch 1, as *S. aureus* is a name conserved by the Judicial Commission on Bacteriological Nomenclature (1958), with strain NCTC 8532 (no. 4 in this paper) as neotype. The correct name for group (*b*), called here *S. saprophyticus*, is in doubt for the group corresponds to *S. epidermidis* (Winslow & Winslow) Evans in *Bergey's Manual* (1957).

The name *Micrococcus* for branch 2 is also of doubtful legality for the Judicial Commission (1958) has not yet considered the validity of the name *Micrococcus luteus*. However, the name *M. roseus* Flügge is widely used and would be convenient for group (*c*) in the Adansonian classification, with strain NCTC 7523 (type strain for *Staphylococcus roseus* Tavel proposed by Shaw *et al.*) as neotype.

It seems premature to recognize *Staphylococcus lactis* and *S. afermentans* as species, for the Adansonian classification shows them to be artificial groups. This is not an unexpected result, because both species were defined by Shaw *et al.* (1951) mainly upon negative features. The essential features given for *S. lactis* were coagulase-negative, acid from glucose, Voges-Proskauer negative and the absence of pink pigment. There were only two essential features for *S. afermentans*, coagulase-negative and acid not produced from glucose. That this is not sound taxonomy is shown up in the Adansonian classification in which each strain of these 'species' constitutes a separate group, equal in status to the three previously named species. Unless one is particularly interested in one of these strains their nomenclature could be limited to simply 'miscellaneous staphylococci (fermentative)' or 'miscellaneous micrococci (non-fermentative).' Strains 45 and 48 are properly called intermediate, whilst strain 29 is one of the miscellaneous staphylococci rather more irregular than the majority.

An identification key can be drawn up for recognizing five groups only as shown below:



The characters chosen are those which are most constant in these groups. If a strain should happen to be aberrant in one of these characters it would be misclassified if this key were used alone, but it should be possible to identify such strains if they are checked with the characters given in Table 2. Thus, if a typical strain of the *Staphylococcus aureus*, *S. saprophyticus* or *Micrococcus roseus* is encountered it can be readily recognized on a few tests only. If a strain does not fit into any of these groups, even after checking with Table 2, then it can be described as simply fermentative or non-fermentative (plate technique). This indicates to which genus the strain probably belongs, but only a full investigation of its properties will reveal its true taxonomic position.

Table 2. *General properties of the three recognizable species*

Property	*S. aureus*	*S. saprophyticus*	*M. roseus*
Golden yellow	Usually +	Usually −	−
White	Usually −	Usually +	−
Pink	−	−	+
Free coagulase	+	−	−
α-lysin	Usually +	−	−
Phosphatase	+	Often +	−
V.P., M.R.	Usually +	Usually +	−
Mannitol	Usually +	Often +	−

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REFERENCES

Abd-el-Malek, Y. & Gibson, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

Barber, M. & Kuper, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

Bergey's Manual of Determinative Bacteriology (1948). 6th ed. Ed. by Breed, R. S., Murray, E. G. D. & Hitchens, A. P., p. 235. London: Baillière, Tindall and Cox.

Bergey's Manual of Determinative Bacteriology (1957). 7th ed. Ed. by Breed, R. S., Murray, E. G. D. & Smith, N. R., p. 454. London: Baillière, Tindall and Cox.

Duthie, E. S. (1954) Evidence of two forms of staphylococcal coagulase. *J. gen. Microbiol.* **10**, 427.

Elek, S. D. & Levy, E. (1950*a*). Diffusible antigens in staphylococcal cultures. *Brit. J. exp. Path.* **31**, 358.

Elek, S. D. & Levy, E. (1950*b*). Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. *J. Path. Bact.* **62**, 541.

Judicial Commission on Bacteriological Nomenclature (1958). *Int. Bull. bact. Nomen. & Taxon.* (in the Press).

Lack, C. H. (1957). Plate coagulase and fibrinolysin tests for staphylococci. *J. clin. Path.* **10**, 208.

Mackie, T. J. & McCartney, J. E. (1953). *Handbook of Practical Bacteriology*, 9th ed. p. 428. Edinburgh: E. and S. Livingstone Ltd.

Marks, J. (1952). Recognition of pathogenic staphylococci, with notes on non-specific staphylococcal haemolysins. *J. Path. Bact.* **64**, 175.

Parker, M. T. & Lapage, S. P. (1957). Penicillinase production by *Staphylococcus aureus* strains from outbreaks of food-poisoning. *J. clin. Path.* **10**, 313.

Shaw, C., Stitt, J. M. & Cowan, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

Sneath, P. H. A. (1956). Cultural and biochemical characters of the genus *Chromobacterium*. *J. gen. Microbiol.* **15**, 70.

Sneath, P. H. A. (1957*a*). Some thoughts on bacterial classification. *J. gen. Microbiol.* **17**, 184.

Sneath, P. H. A. (1957*b*). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

Wilson, G. S. (1955). Symposium discussion on the Principles of Microbial Classification. *J. gen. Microbiol.* **12**, 321.

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The Use of Models in Bacterial Classification

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SUMMARY: The making of models for taxonomic purposes and their use is described, and the fidelity with which they can represent the taxonomic relations between bacteria is discussed. These models are particularly useful for teaching purposes.

The use of equally weighted features in creating taxa allows the comparison of organisms by simple mathematical methods in which the similarity between two strains can be expressed as a percentage; the strains can then be grouped into taxa whose members show high similarity with each other. This method was described by Sneath (1957*a*, *b*), who used it for a study of the genus *Chromobacterium*. It also permits solid models to be made of taxonomic groups, in which the spatial arrangements of the component parts of a model is an expression of the relation of organisms or groups of organisms within the taxonomic group. The preparation of such models is the subject of the present paper.

METHODS

The meaning of the symbols used is given in Sneath (1957*b*). Two groups of bacteria were used for the models illustrated here. The first was the family Enterobacteriaceae as it is at present generally defined. The second was the group of chromobacteria studied by Sneath (1957*b*).

Enterobacteriaceae. In Table 1 are given the features currently used for the differentiation of a number of groups of the Enterobacteriaceae under names which are commonly given them. From Table 1 values of S were calculated by the method of Sneath (1957*b*); these values are shown in Table 2. Since the data in Table 1 do not represent the features of single strains, a slightly different convention for obtaining S was used; +, (+) and × were counted as positive, and d was counted as NC (no comparison). The data in Table 1 were taken mainly from the papers by Cowan (1956) and Kauffmann (1956), with supplementary information from Møller (1955) and Davis, Ewing & Reavis (1957).

It should be noted that the tests used are selected, and therefore are biased; the use of these tests cannot show whether the groups are valid taxa, since this prejudges the question. They are used only as an illustration of the use of models, and the model is not a model of the ideal taxonomy of the family, but only of the current ideas about the taxonomy of the family. The model shown (Pl. 1, figs 1, 2) was made with distance d proportional to $1-S$ (see below).

Table 1. *Differential characteristics of Enterobacteriaceae*

Group name	No.*	Motility	Gas from glucose	Lactose	Sucrose	Mannitol	Adonitol	Dulcitol	Inositol	Sorbitol	Salicin	Maltose	Arabinose	Xylose	Rhamnose	Raffinose	Trehalose	Gelatin	H_2S	Urease	Indole	MR	VP	Citrate	Glucose utilization	KCN	Phenylalanine	Malonate	Lysine	Arginine	Ornithine	Glutamic acid
Escherichia coli	1	+	+	+	d	+	−	d	−	+	d	+	+	+	+	d	+	−	−	−	+	+	−	−	+	−	−	−	+	+	+	+
Salmonella	2	+	+	−	−	+	−	+	d	+	−	+	+	+	+	−	d	−	+	−	−	+	−	+	+	−	−	−	+	+	+	−
Arizona	3	+	+	d	−	+	−	−	−	+	−	+	+	+	+	−	+	+	+	−	−	+	−	+	+	−	−	+	+	+	+	−
Citrobacter freundii	4	+	+	+	d	+	−	d	−	+	d	+	+	+	+	d	+	−	+	−	−	+	−	+	+	+	−	d	−	+	d	−
Cloaca	5	+	+	d	+	+	d	−	d	+	d	+	+	+	+	d	+	+	−	d	−	−	+	+	+	+	−	d	−	+	+	−
Klebsiella	6	−	+	+	+	+	+	d	+	+	+	+	+	+	+	d	+	−	−	−	−	−	+	+	+	+	−	+	+	−	−	−
Hafnia	7	+	+	−	d	+	−	−	−	−	d	+	+	+	+	−	+	−	(+)	−	−	−	+	+	+	+	−	+	+	−	+	−
Serratia	8	+	d	−	+	+	d	−	d	−	+	+	−	d	−	−	+	+	d	d	−	−	+	+	+	+	−	−	+	−	+	−
Proteus vulgaris	9	+	+	−	+	−	−	−	−	−	+	+	−	+	−	−	×	+	+	+	+	+	−	×	×	+	+	−	−	−	−	(+)
P. mirabilis	10	+	+	−	×	−	−	−	−	−	d	−	−	+	−	−	+	+	+	+	−	+	d	×	×	+	+	−	−	−	+	(+)
Morganella	11	+	+	−	d	−	−	−	−	−	−	−	−	−	−	−	d	−	(+)	+	+	+	−	−	−	+	+	−	−	−	+	+
Providencia	12	+	d	−	d	−	d	−	d	−	d	−	−	−	−	−	d	−	−	−	+	+	−	d	d	+	+	−	−	−	−	+
Rettgerella	13	+	d	−	d	−	+	−	+	−	d	−	d	−	d	−	−	−	−	+	+	d	−	+	d	+	+	−	−	−	−	+
Shigella type A 1†	14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+

Symbols: + = prompt positive; (+) = delayed positive; × = late, irregular or mutative positive; d = different reactions with different strains; − = negative.

* No. = numbering of organism in model (Pl. 1, figs. 2, 3).

† The Shigella chosen was Shiga's bacillus, since the group is a very heterogeneous one.

Table 2. *S values of Enterobacteriaceae*

Table 2 was derived from Table 1 as follows: for a particular comparison between two bacteria, $S = n_s/(n_s+n_d)$, where n_s is the number of features for which they are both positive, and n_d is the number of features for which one is positive and the other negative (and vice versa). The entries +, (+) and × in Table 1 were counted as positive, − was negative and d was counted as NC (no comparison made).

		Group no.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		% *S* values													
Group name	No.														
Escherichia	1	100													
Salmonella	2	73	100												
Arizona	3	70	88	100											
Citrobacter	4	66	81	83	100										
Cloaca	5	55	75	70	76	100									
Klebsiella	6	46	45	50	60	74	100								
Hafnia	7	59	63	70	66	72	65	100							
Serratia	8	39	40	47	43	69	55	66	100						
Proteus vulgaris	9	42	35	41	50	47	34	42	50	100					
P. mirabilis	10	36	36	47	47	47	28	45	50	81	100				
Morganella	11	32	24	23	26	20	8	25	17	50	61	100			
Providencia	12	27	12	11	20	12	6	12	20	38	45	67	100		
Rettgerella	13	17	11	11	17	20	23	16	21	46	43	54	83	100	
Shigella type A1	14	24	11	15	18	11	5	5	6	16	19	17	25	8	100

Group of chromobacteria. The *S* values were taken from Sneath (1957*b*, Table 7). These values are based on a wide range of tests in which the bias was as far as possible eliminated, and they therefore represent taxonomic relations which are considered to be valid. The model shown (Pl. 2, figs. 3, 4) was made with distance *d* proportional to $(1/S)-1$ (see below).

Conversion of S *values into distance in the models.* To convert *S* values into distances several different conventions are possible. The simplest is to make the distance, *d*, between two strains proportional to $1-S$, when *S* is the *S*-value between the two strains. This has the disadvantage that parts of the model may be separated by distances which represent a smaller similarity than 0 %. To avoid this one may make the distance between strains proportional to $(1/S)-1$, since the distance which corresponds to 0 % similarity is infinity; this convention exaggerates the low similarities. Other conventions, such as making the distance proportional to the logarithm of $(1/S)$ may be used. We do not consider that any of these conventions has any special taxonomic significance or validity; they are simply conventions which are useful for certain purposes. The convention which probably best represents taxonomic relations is a form of the Generalized Distance (see Rao, 1948) in which generalized distance is equal to n_d in the symbols of Sneath (1957*b*). This is a convention which uses multidimensional Euclidean space, but since the distance *d* depends on the number of features examined, it must for useful results be converted into some form of relative distance which is theoretically independent mathematically of the number of features studied. There are other difficulties in using this convention, such as the uncertainty of the distance *d* which is produced by NC entries in the data; we have therefore not applied it in the present paper.

Form of the models. The most convenient materials are rubber balls held together by lengths of brass rod (brass pinwire, 1–2 mm. diameter). Cork balls may be used but they are apt to break up. Soft copper or iron wire is too pliable for use. The pinwire was cut into lengths determined by the S values between the bacteria and the convention employed for representing S. The ends of the pinwire were pushed to the centre of the balls. Sometimes when the balls are very close it is necessary to slice away part of them to get their centres the correct distance apart. Each bacterium (each group of enterobacteria in the first model, or each strain in the second model) was represented by one ball. It was found convenient to choose three or four well-separated bacteria as a locus around which to add the other bacteria. As mentioned in the Discussion, some distortion of the S values is inevitable in making a model; this was kept to a minimum by trial and error during the building of the models.

RESULTS

Enterobacteria. It is seen from Pl. 1, figs 1, 2, that the model may be divided into three main parts: (1) that containing *Proteus sensu latu*; (2) that containing Shiga's bacillus; (3) that containing the remainder of the groups. It seems very probable, in view of the intermediate forms which occur between *Shigella* and *Escherichia* and the fact that they can undergo genetic exchange (Luria & Burrous, 1957), that the other members of *Shigella* would be interforms connecting Shiga's bacillus to group (3) if they were included in this model.

The close relation of *Salmonella* to *Arizona* is well shown, as are the close relations of *Proteus vulgaris* to *Proteus mirabilis*, and of *Rettgerella* to *Providencia.* These relations are not unexpected, since this is a model of current ideas of the taxonomy of the family, and not necessarily of the best general taxonomy. In Table 3 are given the Cartesian co-ordinates of this model, from which it can be reconstructed.

In the chromobacteria (Pl. 2, figs. 3, 4) it is seen that there are two large but distinct clusters of balls, representing the mesophilic and the psychrophilic groups of *Chromobacterium.* Aberrant and atypical strains are represented by outlying balls. The other strains are chromogenic bacteria which are distinct from the genus *Chromobacterium* itself. At the top are two organisms which seem more closely allied to the Gram-positive bacteria. Strains of *Pseudomonas* and of *Serratia* are also shown. *Serratia* is represented in both models, and this shows roughly how the enterobacteria and chromobacteria are related to one another.

If we had used a number of strains of each group of the enterobacteria we would have obtained clusters of balls which would be centred around the points at which the single balls are present in the first model. These single balls represent typical strains (modoforms) of the groups. It is likely that the clusters would have overlapped a good deal.

Table 3. *Cartesian co-ordinates of the model of enterobacteria*

These co-ordinates are the distances in cm. of the balls in the model (Pl. 1, figs. 2, 3) given in three axes at right angles to one another. The origin is the ball 6, representing Klebsiella.

		Distance from the origin in the three axes x, y, z		
		x	y	z
Group name	No.	Distances (cm.)		
Escherichia	1	0	5·9	0
Salmonella	2	3·2	4·2	0
Arizona	3	3·7	5·9	1·0
Citrobacter	4	2·6	4·8	2·0
Cloaca	5	3·1	2·2	1·4
Klebsiella	6	0	0	0
Hafnia	7	2·8	0·1	2·8
Serratia	8	4·9	2·6	4·6
Proteus vulgaris	9	1·1	5·0	6·6
P. mirabilis	10	0·6	3·3	5·9
Morganella	11	−2·6	3·3	5·6
Providencia	12	−4·1	7·9	2·6
Rettgerella	13	−4·3	6·4	3·8
Shigella type A1	14	−0·1	12·5	2·2

The distance, s, between two points is given by the formula $d^2 = x^2+y^2+z^2$, where x, y and z are the algebraic differences between the co-ordinates of the two points in the x, y and z axes respectively.

DISCUSSION

The older schemes of bacterial taxonomy may be more of a handicap than a help in the future. A new general taxonomy of bacteria has not yet been developed (though attempts are now being made in this direction; see Sneath & Cowan, 1958), and models such as those shown here may be of help in obtaining a general orientation when building such a taxonomy. One of the greatest difficulties is the lack of comparative bacteriology, since much of our knowledge comes from specialities such as dairy microbiology and soil microbiology, and it seems probable that many bacteria known by different names to workers in different specialities are in fact identical. A classic case is in *Pseudomonas*, where it is very likely that some of the species which are pathogenic for plants are identical with *Pseudomonas aeruginosa* from animals (see Elrod & Braun, 1942). It is also not unusual to be unable to identify a well-known bacterium when it has been isolated from an unusual habitat.

The biological relationships of organisms are of course not the same as the mathematical relations in these models, since the latter are only summaries, incomplete and distorted, of the former. This distortion is inevitable because, as has been mentioned in the description of the method, the relations between organisms are multidimensional, with one dimension for every feature which can vary independently of the rest. This compression, with subsequent distortion, into two dimensions (as in taxonomic trees) or into three (as in the models) is, however, essential for handling and publishing taxonomic data. It would ideally be carried out by the mathematical procedure of Principle Component Analysis.

The value of the models is that they allow better expression of relations than is afforded by two dimensions, they are useful in obtaining a preliminary idea of the taxonomy of a group under study and they are very suitable for teaching. Intermediate strains (interforms) in such models are placed between the clusters which represent the more typical members of the groups under study. One other useful property is that, by colouring or labelling the balls, the reactions of the strains in a few tests which are of special concern to the worker in diagnostic or epidemiological work can strikingly be shown.

The degree to which the *S* values are distorted on reducing them from a multidimensional quantity to one in three dimensions does not seem, in the present examples at any event, to be very great. This was checked in the first model by measuring all the distances between bacteria and comparing them with the *S* value table (the actual distances may be derived from Table 3). The great majority of such distances were close to those implied by the *S* values, though in a few the differences were over 2·5 cm. (corresponding to 25 % *S*). In the best possible model these discrepancies would be as small as possible.

REFERENCES

COWAN, S. T. (1956). Taxonomic rank of Enterobacteriaceae 'groups'. *J. gen. Microbiol.* **15**, 345.

DAVIS, B. R., EWING, W. H. & REAVIS, R. W. (1957). The biochemical reactions given by members of the *Serratia* group. *Internat. Bull. bact. Nomen. Taxon.* **7**, 151.

ELROD, R. P. & BRAUN, A. C. (1942). *Pseudomonas aeruginosa*: its role as a plant pathogen. *J. Bact.* **44**, 633.

KAUFFMANN, F. (1956). A simplified biochemical table of Enterobacteriaceae. *Acta path. microbiol. scand.* **39**, 103.

LURIA, S. E. & BURROUS, J. W. (1957). Hybridization between *Escherichia coli* and Shigella. *J. Bact.* **74**, 461.

MØLLER, V. (1955). Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta path. microbiol. scand.* **36**, 158.

RAO, C. R. (1948). The utilization of multiple measurements in problems of biological classification. *J. R. statist. Soc.* B, **10**, 159.

SNEATH, P. H. A. (1957*a*). Some thoughts on bacterial classification. *J. gen. Microbiol.* **17**, 184.

SNEATH, P. H. A. (1957*b*). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

SNEATH, P. H. A. & COWAN, S. T. (1958). An electrotaxonomic survey of bacteria. *J. gen. Microbiol.* **19**, 551.

EXPLANATION OF PLATES

Plate 1

Fig. 1. Taxonomic model of the Enterobacteriaceae. The numbers and names where shown are those used for the groups in Table 2.

Fig. 2. Taxonomic model of the Enterobacteriaceae: another view.

Plate 2

Fig. 3. Taxonomic model of chromobacteria. The groups of strains were as follows: (*a*) = mesophilic strains of *Chromobacterium* (*C. violaceum*); (*b*) = psychrophilic strains of *Chromobacterium* (*C. lividum*); (*c*) = *Serratia*; (*d*) = *Pseudomonas aeruginosa*; (*e*) = 'Chromobacterium viscosum'; (*f*) = 'Chromobacterium iodinum'; (*g*) = 'Chromobacterium ianthinum'.

Fig. 4. Taxonomic model of chromobacteria: another view.

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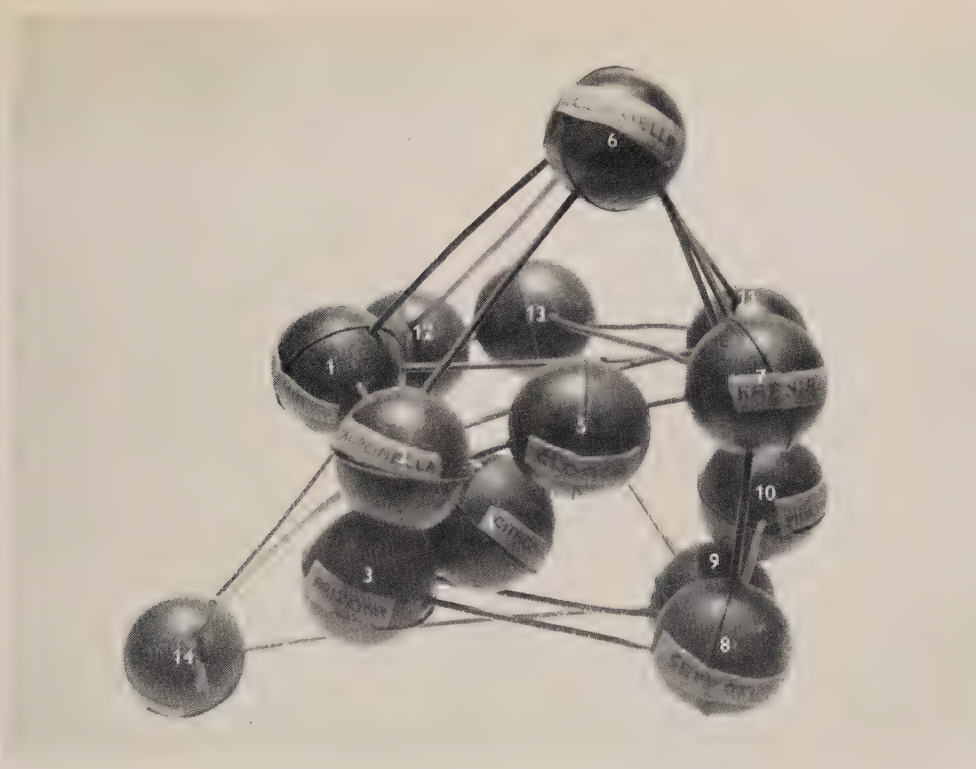
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Fig. 1

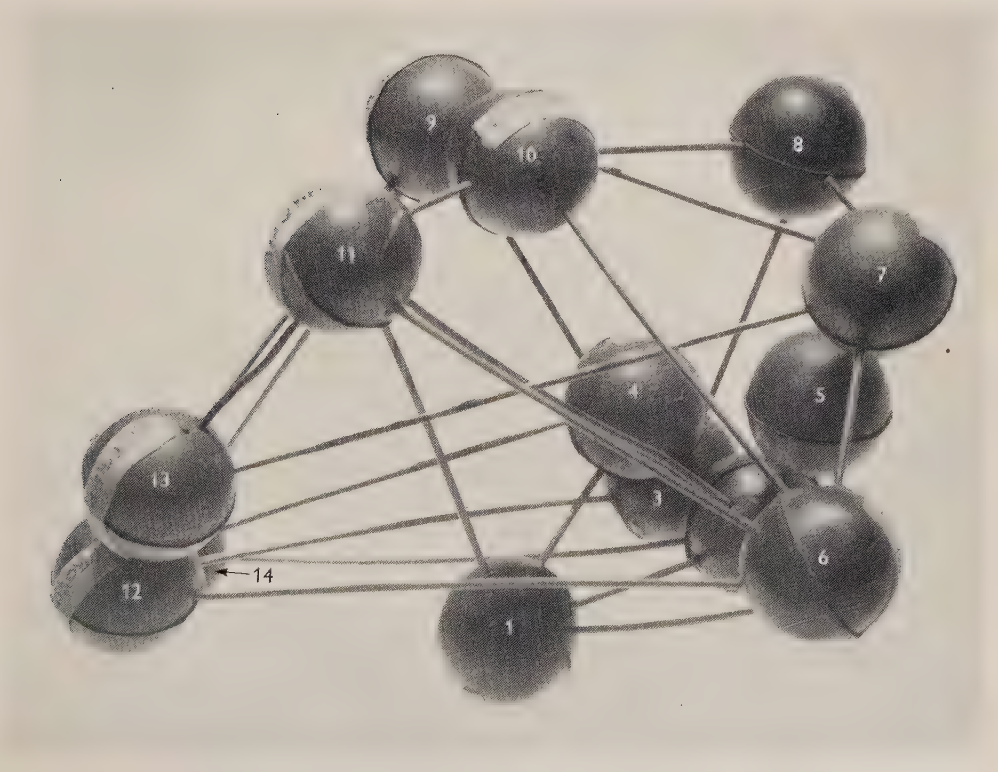
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Fig. 2

O. Lysenko & P. H. A. Sneath—Models in taxonomy. Plate 1

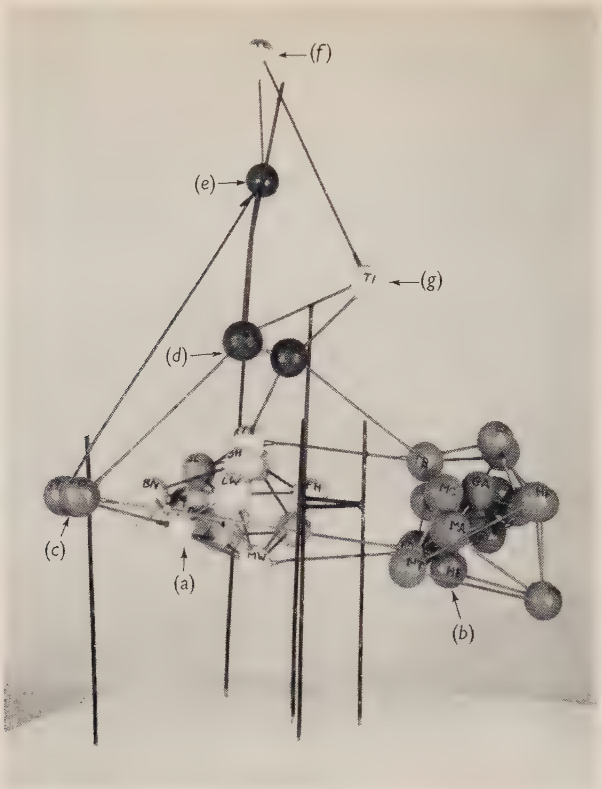
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Fig. 3

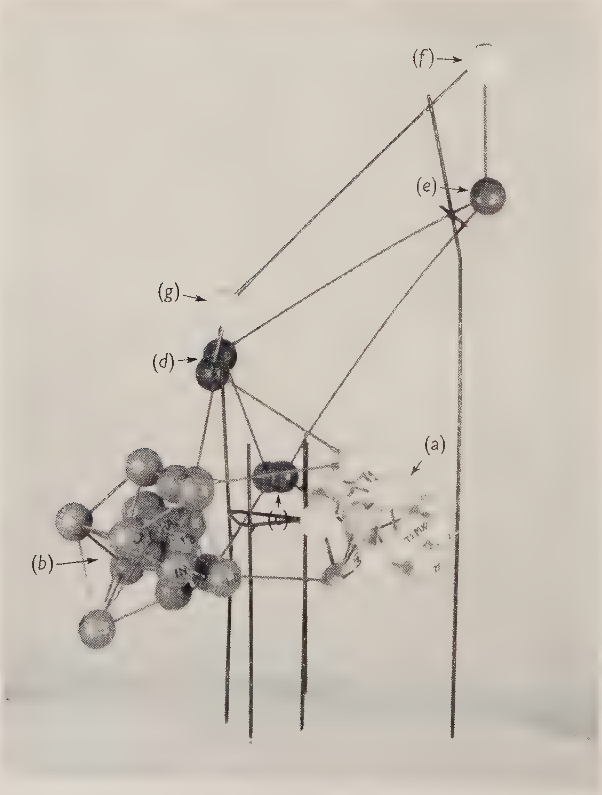

Fig. 4

O. Lysenko & P. H. A. Sneath—Models in taxonomy. Plate 2

VAN HEYNINGEN, W. E. (1959). *J. gen. Microbiol.* **20**, 291–300

The Fixation of Tetanus Toxin by Nervous Tissue

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SUMMARY: The fixation of tetanus toxin by nervous tissue (Wassermann–Takaki phenomenon, 1898) has been re-investigated. Fixation is greatly dependent on the concentration of toxin and of receptor. Toxin is fixed in preference to toxoid, but its toxicity does not appear to be markedly diminished by fixation. A biological assay of the toxin receptor in nervous tissue and its components has been devised. The receptor activity is not due to a cerebroside, as thought by Landsteiner & Botteri (1906), but to some substance (present mainly in grey matter) that tends to associate with it.

Tetanus toxin acts primarily on the central nervous system, although it appears also to have at least some peripheral action (see Wright, 1955). Its action on the central nervous system has been shown by Brooks, Curtis & Eccles (1955) to consist in suppressing synaptic inhibition, possibly either by preventing the release of the transmitter substance from the presynaptic terminal of the interneurone, or its access to the subsynaptic membrane of the motor neurone. In order to determine the biochemical lesion underlying this intervention it is necessary to identify the substance at the inhibitory synapse upon which the toxin acts, to study the action of the toxin on this substance, and to determine the consequences which follow immediately upon this action. It seemed possible that a re-investigation of the well known Wassermann–Takaki phenomenon might be a fruitful way of approaching the problem. Wassermann & Takaki (1898) showed that when an emulsion of brain tissue was mixed with a few lethal doses of toxin the toxicity was decreased, and the decrease of toxicity was greater when the mixture was filtered before injection. Evidently the toxin was fixed on nervous tissue. Brain tissue was more effective than spinal cord, and grey matter more than white. This phenomenon attracted much attention during the following decade, and many workers attempted to identify the material in nervous tissue responsible for the fixation. The most effective work was that of Landsteiner & Botteri (1906; see also Takaki, 1908), who showed that the 'protagon' component of nervous tissue had the greatest toxin receptor activity. Protagon is the component which is extracted from nervous tissue with hot ethanol after previous exhaustive extraction, first with acetone, then with ether; it is a mixture of sphingolipids, particularly the cerebrosides and sphingomyelins. Landsteiner & Botteri and Takaki believed that the cerebroside phrenosine had powerful receptor activity, but were puzzled by the fact that whereas grey matter showed much greater receptor activity than white matter, it had a much smaller phrenosine content. Interest in the phenomenon then died out, and during the next fifty years little work was done on it.

The phenomenon seemed to be worth further study, however, because of its specificity. Thus it had been shown by Coleman (1924) not to apply to botulinus and diphtheria toxins, by Fulthorpe (1956) not to apply to diphtheria, *Clostridium oedematiens*, *C. welchii* and staphylococcus toxins, and by Pons (1938) and Fulthorpe (1956) not to apply to tetanus toxoid. This suggested that there might be a significant relationship between tetanus toxin and some component of the grey matter of nervous tissue. Since the inhibitory synapses are present in the grey matter it is reasonable to search for the susceptible substance of tetanus toxin in this tissue. It was decided therefore to confirm that the fixation is specific for tetanus toxin, to determine whether it is due to a particular component of nervous tissue, and if so, what this substance is, and how the toxin reacts with it. The first step was the development of a quantitative assay for the toxin receptor activity of nervous tissue and its various components.

METHODS

Toxin. The tetanus toxin preparation, XW 1322, was kindly supplied by Miss Mollie Barr of the Wellcome Research Laboratories. It was a dried precipitate obtained by saturating a culture filtrate of *Clostridium tetani* with $(NH_4)_2SO_4$. The powder consisted of 13·5 % protein (Lowry, Rosebrough, Farr & Randall, 1951) mixed with salts containing much $(NH_4)_2SO_4$. It contained 430,000 LD 50 doses (mice), 15·7 L+ doses and 18 Lf doses/mg. dry weight, or 3,185,000 LD 50, 116 L+, and 133 Lf/mg. protein, i.e. 27,350 LD 50/L+ and 1·146 Lf/L+. Comparison with the purified toxin of Pillemer, Wittler, Burrell & Grossberg (1948, $6{\cdot}6 \times 10^7$ MLD/mg. N; 3400–3600 Lf/mg. N; 15·7 % N) shows that the preparation XW 1322 was about 25 % pure (leaving aside its salt content), and somewhat less toxoided. There was no advantage in using pure toxin for this work, even if it could be prepared or were available, because the pure toxin readily denatures and spontaneously toxoids (Pillemer *et al.* 1948); in any case the pharmacological action of the toxin is so distinctive that there was no pressing need to separate it from irrelevant materials. The preparation of antitoxin, containing 1850 units/ml., was also supplied by Miss Mollie Barr.

Assay of toxin. Toxin and antitoxin were dissolved in 0·067 M-phosphate buffer (pH 7) containing 0·3 % (w/v) gelatin. In determinations of LD 50 dose and L+ dose (at 0·1 antitoxin unit level), a series of 0·5 ml. volumes containing 1·25-fold dilutions (20 % steps) of toxin (mixed with 0·1 unit of antitoxin in the case of L+ determinations) was injected subcutaneously into the hind limbs of 16–20 g. mice. Four to eight mice were used for each dilution of toxin and generally seven concentrations were used, to cover a four-fold range. Values for LD 50 and L+ were calculated from the deaths recorded in 7 days.

Figure 1 shows the smooth dose-response curves drawn through the data derived from several hundred determinations each of LD 50 and L+. They show that 140 % of an LD 50 dose, or 120 % of an L+ dose, killed all the mice injected, and 60 % of an LD 50, or 80 % of an L+, killed none. This meant that assays of toxin could be carried out with reasonable accuracy and with relatively few mice, especially in the presence of antitoxin. In assays of L+

one very seldom finds a dose, even when doses are spaced only 20 % apart, that kills only some of the mice. In most assays either all the mice injected with a given dose die, or all survive; consequently the value or accuracy of the assay is very little affected whether 2 or 8 mice are used per dose.

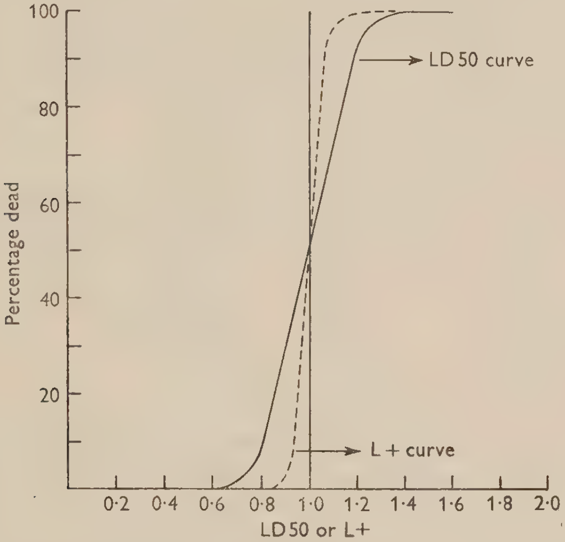

Fig. 1. Smooth dose-response curves drawn through data derived from several hundred determinations of LD50 and L+ (at 0·1 antitoxin unit level).

Determinations of Lf were done at 50°, with the antitoxin concentration in successive tubes falling in 20 % steps. The crude toxin preparation flocculated only in one zone, which was shown by an L+ determination to be due to the toxin.

Fractionation of brain. Fresh or deep-frozen beef brain from the slaughterhouse was freed from blood and meninges under running tap water, minced in a Waring Blendor, and separated into fractions soluble in acetone, ether and hot 85 % (v/v) ethanol in water (protagon) by the method of Loening & Thierfelder (1910). Fresh brain (1·6 kg.) yielded 110 g. acetone-soluble material, 60 g. ether-soluble material, and 28 g. protagon. Protagon was also prepared from chicken brain in the same way. Crude phrenosine (10 g.) was prepared from the beef protagon by the method of Klenk & Leupold (1944) and further purified (6·2 g.) by the method of Carter & Fujino (1956). Samples of pure phrenosine and sphingomyelin from brain were also kindly supplied by Dr H. E. Carter of Urbana and Professor E. Klenk of Cologne.

Protagon, phrenosine and sphingomyelin are insoluble in water, and when suspended in water, and the water brought to the boil and cooled, they form very stable opalescent suspensions showing myelin formation. They can be removed from these suspensions by centrifuging for 1 hr. at 5600 *g* (5000 rev./min., 20 cm. mean diam. angle centrifuge).

RESULTS

Adsorption from low concentrations of toxin

For the assay of the receptor it was decided to apply the same principle as is used in the assay of antitoxin, that is, to determine the least amount of receptor (the Receptor Unit—RU) that would neutralize (in this case by removal by adsorption) a test dose of toxin. The test dose chosen at first was 10 LD 50, since the earlier workers had investigated the effect of brain emulsions on a few lethal doses of toxin. Estimations of the receptor content (RU–10 LD 50/mg.) of brain and its components were carried out as follows. To seven or eight 4 ml. volumes of a series of 1·43-fold dilutions (30 % steps) in water of suspensions of brain, protagon, crude phrenosine and purified sphingomyelin were added equal volumes of toxin solution in gelatin phosphate buffer (pH 7) containing 44 LD 50/ml. After mixing and standing for 2 hr. at 37° the mixtures were centrifuged for 1 hr. at 5600 *g* and 0·5 ml. volumes of the supernatant fluids injected subcutaneously into mice, four mice being used for each mixture. From the deaths observed in 7 days the amount of receptor which adsorbed all but one of 11 LD 50 doses could be calculated. The values thus obtained are recorded in Table 1. This table confirms Landsteiner & Botteri's (1906) observation that the toxin receptor activity of brain lies in its protagon component, and that the crude phrenosine component of protagon is active; and it shows that sphingomyelin, which has many of the physical properties of phrenosine (e.g. myelin formation in water), is inactive. The protagon component, however, had no more receptor activity than the brain from which it was derived, and since it comprises only about 8–10 % of the dry weight of brain its receptor activity might be expected to be about 10 times as great. It was realized then that with a test dose of 10 LD 50 the concentration of toxin used, in terms of weight of toxic protein/unit volume, was extremely low, of the order of 0·002 μg./ml. Fulthorpe (1956) showed that the fixation of tetanus toxin by brain follows the usual laws of adsorption, i.e. the greater the concentration of toxin the greater the amount adsorbed. His experiments were carried out at toxin concentrations varying from 5 to 20 L+/ml., i.e. up to 50,000 times as great as those in the present experiments. It was decided therefore to use comparable concentrations of toxin.

Table 1. *Tetanus toxin receptor activity of brain and its components as measured in a low concentration of toxin* (22 *LD* 50/*ml.*; 0·002 μ*g. toxic protein/ml.*)

Receptor preparation	RU–10 LD 50/mg.	Receptor preparation	RU–10 LD 50/mg.
Whole beef brain (dry weight)	1·7	Extracted brain residue	<0·7
Acetone-soluble fraction	<0·7	Crude phrenosine	1·8
Ether-soluble fraction	<0·7	Sphingomyelin	<0·7
Whole brain protagon	2·2		

Adsorption from high concentrations of toxin

Flocculation studies. At concentrations about 20 L+ or Lf/ml. toxin flocculates rapidly with equivalent concentrations of antitoxin, and it was hoped therefore to estimate receptor activity rapidly and *in vitro* by measuring the Lf content of a toxin solution before and after treatment with a receptor preparation. However, Table 2 shows that when this was done there was a sharp decrease in the L+ content but no decrease in the apparent Lf content, although there was an increase in flocculating time (Kf). This observation is reminiscent of that of Fulthorpe (1956), who found that on two successive adsorptions of toxin with dried horse brain there was a parallel decrease in L+ and Lf contents, but on the third adsorption, when the L+ content diminished to 70 %, the apparent Lf content doubled. There seems to be no explanation for these anomalous results, except possibly that the toxin preparation contained two antigens, one toxic and the other irrelevant, flocculating in the same zone at different times. In any case, as will be seen in the next section, there is no doubt that the toxin is adsorbed on the receptor.

Table 2. *Apparent failure of protagon to remove flocculating antigen while removing toxin*

	Assays after treatment of toxin preparation with protagon		
mg. protagon/ml.	L+/ml.	Apparent Lf/ml.	Kf
0	52	60	480
0·25	42	55	923
1	12	44	806
3·8	2	60	1888
7·5	0	60	1120
15	0	60	1152

Kf = units antitoxin/ml. × flocculation time (min.).

Toxicity of toxin-receptor complex. Table 3 shows that when a suspension of receptor with toxin adsorbed on it was injected into mice the action of the adsorbed toxin was unimpaired and could be accounted for quantitatively.

Table 3. *Quantitative recovery of toxin adsorbed on protagon*

10 ml. Toxin-protagon mixture containing 100 L+ toxin and 10 mg. protagon, stood for 2 hr. at 37° and centrifuged

10 ml. supernatant fluid assayed for L+ content: 27 L+ doses.

Residue suspended in 10 ml. buffer and assayed for L+ content: 77 L+ doses.

At low concentrations the action of toxin was somewhat diminished when injected together with receptor. Thus, while 1 mg. of a protagon preparation sufficed to protect mice against 10 LD50 of toxin when it was centrifuged out before injection, 4 mg. of protagon were needed when injected together with the toxin. The toxicity of the toxin-protagon complex may be due to dissociation on dilution in the body fluids of the mice, since the lower the toxin concentration the less is adsorbed. This suggestion was supported by the finding that when protagon and toxin at a concentration of 30 L+/ml. were stood for 2 hr., 0·64 mg. protagon were required to fix 20 L+ units of toxin (see section below on assay of receptor), but when the same mixtures, after standing at 30 L+/ml. for 2 hr., were diluted 1/40, an equivalent of 8·5 mg. protagon were required to adsorb 20 L+ doses.

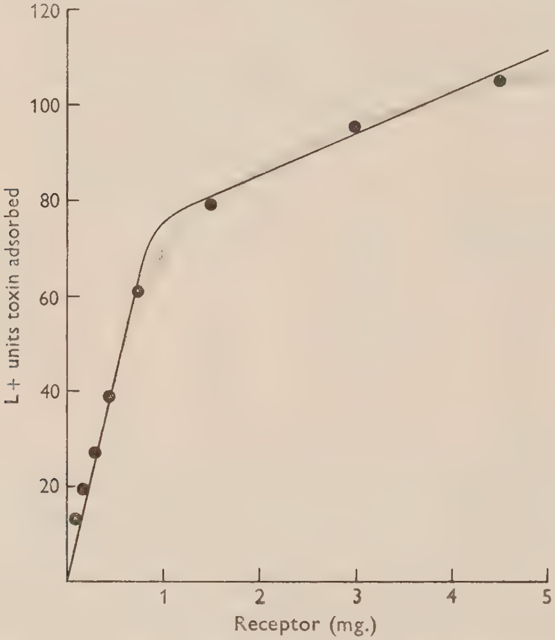

Fig. 2. Effect of increasing concentration of receptor on adsorption of toxin.

Effect of pH value, temperature and salt concentration on adsorption of toxin. Experiments showed that adsorption of toxin by protagon decreased rapidly at values of pH above 7. It was not possible to test adsorption at values of pH below 7 because the toxin precipitated. Adsorption was the same at 10° as at 37°, and was somewhat inhibited in high concentrations of salt (in 8 % NaCl adsorption was 63 % of that in 0·25 % NaCl.).

Effect of receptor concentration on adsorption of toxin. Figure 2 shows the adsorption of toxin at constant concentration (30 L+/ml.) by increasing concentrations of protagon (0·0125–0·75 mg./ml.). Adsorption was measured by difference between the L+ values of the solutions before and after treatment with protagon in the usual way. The adsorption curve shows a marked change in slope for which no explanation is forthcoming. Table 4 shows that protagon selectively adsorbed toxin rather than toxoid, since the LD 50/L+

Table 4. *Decrease in LD 50/L+ ratio of toxin solution after treatment with 0·3 mg. protagon/ml. (selective adsorption of toxin)*

	L+/ml.	LD 50/ml.	LD 50/L+
Toxin before adsorption	30	820,500	27,350
Residual toxin after adsorption	6·85	43,500	6,350

ratio of residual material after adsorption was much smaller than that of the preparation before adsorption.

Assay of receptor. In applying the information displayed in Fig. 2 to the assay of the receptor it was clearly advisable to work on the steeper part of the curve, i.e. to determine the amount of receptor required to adsorb a proportion (not more than two-thirds) of a given amount of toxin, rather than the total. It was decided, therefore, to work at a toxin concentration of 30 L+/ml., and to determine the concentration of receptor (RU–20 L+/ml.) that would fix 20 L+/ml., i.e. two-thirds of the total concentration of toxin. This proportion should lie near the top of the steeper part of the curve. The assays were done as follows. Seven or eight 0·5 ml. volumes of a series of 1·43-fold dilutions (30 % steps) in water of suspensions of brain and its various components were mixed with equal volumes of toxin solution in gelatin phosphate buffer (pH 7) containing 60 L+/ml. After standing for 2 hr. at room temperature the mixtures were centrifuged for 1 hr. at 5600 *g*, and 0·5 ml. of each supernatant fluid (containing the unadsorbed residue of 15 L+) mixed with 2 ml. of solution of antitoxin in gelatin phosphate buffer (pH 7) containing 2·5 units/ml. (i.e. 5 units antitoxin). Samples (0·5 ml.) from each mixture were then injected into two mice. Thus in effect the receptor was assayed against 30–10 L+, and the unadsorbed toxin was assayed by L+ assay at the one unit level, which is more accurate than a direct assay of toxicity (e.g. see Fig. 1). It was found that two mice were quite sufficient for each dilution of receptor (i.e. 14 mice for 7 dilutions covering an eightfold range) since the same answers were invariably obtained when more than two mice were used. Moreover, the mice were not observed for more than 3 days, because those which received toxin received such enormous doses that they always died in 3 days. Deaths after 3 days were always adventitious.

The significance of the change of slope in Fig. 2, and the advantage of a test dose that is two-thirds of a given amount of toxin, is shown by the observation that although the weight of the Receptor Unit of a certain preparation of protagon was 0·58 mg. when measured against 30 L+ doses of toxin + 10 units antitoxin, it was 6·0 mg. when measured against 20 L+ alone.

Table 5. *Effect of concentration of toxin on receptor activity of protagon*

Conc. toxin (L+/ml.)	RU–20 L+/ mg. protagon
30	1·6
60	4·3
120	10·0

Table 5 shows that the weight of the Receptor Unit decreased with increasing toxin concentration, as might be expected from Fulthorpe's (1956) observation that the higher the toxin concentration the greater the amount of toxin adsorbed. However, the greater consumption of toxin and antitoxin did not justify carrying out the assay at concentrations of toxin above 30 L+/ml.

Table 6 shows that this method of assay gave satisfactorily reproducible results. Table 7 shows the results of assays on various components of brain. The early observation that grey matter has more receptor activity than white is confirmed, and protagon from grey matter is shown to be more active than protagon from white matter or spinal cord. The data on chicken brain and the protagon derived from it are included because the chicken is extremely resistant to tetanus toxin. Metchnikoff (1898) claimed that chicken brain did not fix the toxin; evidently this claim cannot be confirmed. Comparison with Table 1 shows that when receptor activity was assayed against 500,000 LD 50 rather than 10 LD 50 (or 40 μg. toxic protein rather than 0·001 μg.) a big difference was observed in the relative receptor activities of protagon and brain. Protagon and crude phrenosine were now about 40 times as active as brain. When phrenosine was further purified, however, it lost its receptor activity. Other cerebrosides, and sphingomyelin, also did not have appreciable activity.

Table 6. *Reproducibility of biological assays of receptor activity of a particular preparation of protagon determined on a number of occasions*

Time after first assay (days)	RU–20 L+/ mg. protagon	Time after first assay (days)	RU–20 L+/ mg. protagon
0	2·2	23	2·0
0	2·2	42	1·8
7	2·0	63	2·2
9	2·2	74	2·0
21	1·7		

Table 7. *Tetanus toxin-fixing capacity (RU–20 L+/mg.) of brain and its various components*

Receptor material	RU–20 L+/mg.	Receptor material	RU–20 L+/mg.
Whole beef brain (dry wt.)	0·05	White matter protagon	0·3
Grey matter	0·12	Spinal cord protagon	<0·25
White matter	0·017	Crude phrenosine	1·7
Acetone-soluble fraction	<0·03	Purified phrenosine	<0·2
Ether-soluble fraction	<0·03	Purified mixed cerebrosides	<0·2
Extracted residue	0·03	Sphingomyelin	<0·03
Whole brain protagon	2·2	Whole chicken brain (dry wt.)	0·10
Grey matter protagon	3·3	Chicken brain protagon	2·0

Destruction of toxin by psychosine sulphate

Removal of the fatty acid from cerebrosides by alkaline hydrolysis gives rise to sphingosylgalactose (psychosine) from which a water-soluble sulphate can be prepared. Drury *et al.* (1936) showed that psychosine sulphate inactivated tetanus toxin but not other toxins. This observation has been confirmed in so far as a solution containing 150 μg. psychosine and 20 LD 50 toxin/ml. was found to be non-toxic. This inactivation is irreversible, however, and is probably due to denaturation of the toxin since psychosine sulphate is highly surface-active; a solution of it froths vigorously. It is unlikely that there is any connexion between the adsorption of toxin by protagon and its inactivation by psychosine sulphate.

DISCUSSION

The assays at both low (against 10 LD50 doses) and high (against 20 L+ doses) concentrations of tetanus toxin yielded qualitatively similar results, showing that the receptor activity of nervous tissue is in the protagon fraction and in no other fraction. However, we must consider the anomalous finding that at low toxin concentrations the receptor activity of the protagon fraction was not greater than that of whole brain, whereas at high toxin concentrations it was much greater. A possible explanation of this may lie in the well-known curvature of the adsorption isotherms of heterogeneous systems at low concentrations of adsorbate. At the excessively low concentration of toxin ($2{\cdot}6 \times 10^{-11}$ M) used in the assay against 10 LD50 doses slight differences in the curvature for brain and protagon might account for the anomaly.

The biological method for the assay of the tetanus toxin receptor, in comparison with many biological assays, is reasonably reproducible, accurate and quick, especially since a good idea of the final answer can be obtained on the day after injection. It might be advisable in the future to assay against 10 L+ units of toxin (30 L+ doses of toxin + 20 units of antitoxin) rather than 20 L+ units (30 L+ doses of toxin + 10 units antitoxin) since the point for two-thirds adsorption of toxin lies perhaps too near the top end of the steep part of the curve in Fig. 2.

The demonstration that crude phrenosine has much receptor activity and purified phrenosine practically none, solves the problem which faced Landsteiner & Botteri (1906) who believed that phrenosine was the receptor, but could not reconcile this with the fact that the phrenosine contents of grey and white matter were inversely proportional to their capacity to fix toxin. Evidently the receptor is a substance, mainly present in grey matter, which tends to associate with cerebrosides but is distinct from them. All attempts to separate such a substance from protagon by solvent extraction, adsorption on columns, etc., were unsuccessful, and it was decided to investigate the potentialities of counter-current distribution between immiscible solvents. This technique would require many hundreds of assays of receptor activity, and although the biological assay had been useful up to this stage it was too inaccurate, too laborious, too slow and too expensive to use on such a large scale. It was essential to devise a more practicable assay that could be carried out *in vitro*. This was examined in the following paper (van Heyningen, 1959).

REFERENCES

Brooks, V. B., Curtis, D. R. & Eccles, J. C. (1955). The mode of action of tetanus toxin. *Nature, Lond.* **175**, 120.

Carter, H. E. & Fujino, Y. (1956). Biochemistry of the sphingolipides. IX. Configuration of cerebrosides. *J. biol. Chem.* **221**, 879.

Coleman, G. E. (1924). Action of leucocytes and of brain tissue on toxin of *B. botulinus*. *J. infect. Dis.* **34**, 614.

Drury, A. N., Miles, J. A. R., Platt, A. E., Plaut, G. & Weil, H. (1936). Observations relating to psychosine sulphate: Its action upon bacteria, toxins, serum and red blood cells. *J. Path. Bact.* **42**, 363.

Fulthorpe, A. J. (1956). Adsorption of tetanus toxin by brain tissue. *J. Hyg., Camb.* **54**, 315.

Klenk, E. & Leupold, F. (1944). Über eine vereinfachte Methode zur Darstellung von phosphorfreien Cerebrosiden und über die als Spaltprodukte auftretenden Fettsäuren. 18. Mitteilung über Cerebroside. *Z. physiol. Chem.* **281**, 208.

Landsteiner, K. & Botteri, A. (1906). Über Verbindungen von Tetanustoxin mit Lipoiden. IV. *Zbl. Bakt.* (*Orig.*), **42**, 562.

Loening, H. & Thierfelder, H. (1910). Über das Cerebron. IV. Mitteilung. *Z. physiol. Chem.* **68**, 464.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

Metchnikoff, E. (1898). Recherches sur l'influence de l'organisme sur les toxines. *Ann. Inst. Pasteur*, **12**, 81.

Pillemer, L., Wittler, R. G., Burrell, J. I. & Grossberg, D. B. (1948). The immunochemistry of toxins and toxoids. VI. Crystallization and characterization of tetanal toxin. *J. exp. Med.* **88**, 205.

Pons, R. (1938). L'anatoxine tétanique est-elle une toxine ayant perdu son pouvoir de fixation sur le système nerveux? *C. R. Soc. Biol., Paris*, **129**, 209.

Takaki, T. (1908). Über Tetanusgift bindende Bestandteile des Gehirns. *Beitr. chem. Physiol. Path.* **11**, 288.

van Heyningen, W. E. (1959). Chemical assay of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.* **20**, 301.

Wassermann, A. & Takaki, T. (1898). Über tetanusantitoxische Eigenschaften des normalen Centralnervensystems. *Berl. klin. Wschr.* **35**, 5.

Wright, G. P. (1955). The neurotoxins of *Clostridium botulinum* and *Clostridium tetani*. *Pharmacol. Rev.* **7**, 413.

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van Heyningen, W. E. (1959). *J. gen. Microbiol.* 20, 301–309.

Chemical Assay of the Tetanus Toxin Receptor in Nervous Tissue

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SUMMARY: The fixation of tetanus toxin by the receptor in nervous tissue is highly specific in so far as tetanus toxic protein (and toxoid to a much smaller extent), and apparently nothing else, is adsorbed from solutions of ionic strength greater than 0·15. At lower ionic strengths certain other proteins may be adsorbed by a mechanism that is probably different. The specific adsorption of toxic protein has been used as the basis of a chemical method of assay of the receptor which depends on the estimation of the amount of protein adsorbed under prescribed conditions by the unknown amount of receptor from a fixed amount of crude tetanus toxin.

In the previous paper (van Heyningen, 1959) reasons were given why it seemed unrealistic to attempt the isolation of the tetanus toxin receptor in nervous tissue unless a rapid, convenient and reasonably accurate assay for the receptor could be devised. This paper describes the development of such an assay. It is based on the premise, and subsequent demonstration, that if the receptor is specific in so far as it will adsorb only tetanus toxin and no other toxin, then it should adsorb only tetanus toxic protein, and no other protein. The adsorbed protein can be estimated chemically, and the amount adsorbed from a fixed concentration of toxin, irrespective of the purity of the toxin, is proportional to the amount of receptor added.

METHODS

The toxin and its assay, the antitoxin, the sphingolipid preparations used and the method for suspending them in water were the same as those described in the previous paper (van Heyningen, 1959). In addition a further preparation of tetanus toxin, TD464D, kindly supplied by Dr C. G. Pope and Dr R. O. Thomson of the Wellcome Research Laboratories, was used for one experiment (see Table 1). This preparation was obtained by growing *Clostridium tetani* in Mueller medium for 2 days, lysing the organisms with 1·0 M-NaCl at 0°, filtering the extract, precipitating the toxin at pH 7·9 with 1·5 M-potassium phosphate, dissolving the precipitate in water, dialysing the solution and freeze-drying. It contained 40·8 % protein and 360 L+doses/mg. protein, and was thus about 75 % as pure as the crystalline preparation of Pillemer, Wittler, Burrell & Grossberg (1948; see also van Heyningen, 1959). Protein was estimated with the Folin-Ciocalteau reagent by the method of Lowry, Rosebrough, Farr & Randall (1951), with salt-free crystalline trypsin as standard. The crystalline trypsin, lysozyme and pepsin were commercial preparations (Armour Laboratories, Eastbourne); tetanus toxoid, *C. welchii* Types A, B, C and D and diphtheria crude toxin preparations were kindly supplied by Miss

Mollie Barr and Mrs Irene Batty of the Wellcome Research Laboratories, Beckenham; the crude dysentery toxin preparation was made by extracting dysentery bacilli at pH 11, and neutralizing, clarifying, dialysing and freeze-drying the extract; purified serum albumin and α- and β-globulins were kindly supplied by Dr R. A. Kekwick of the Lister Institute, London.

All centrifuging of sphingolipid preparations was done for 1 hr. at 5600 *g* (5000 rev./min. in a 20 cm. mean diam. angle centrifuge) in glass centrifuge tubes (cleaned with hot acid) of 6 mm. internal and 7·5 mm. external diameter, 10 cm. long. It was found to be important not to use plastic centrifuge tubes as these tended to adsorb protein.

RESULTS

The receptor adsorbs only protein from crude toxin

Figure 1, curve *a*, shows the ultraviolet absorption curve of the crude tetanus toxin preparation XW 1322 in 0·1 N-NaOH, in which 25 % of the protein is toxin (van Heyningen, 1959). The curve is typical of a crude preparation of protein. Curve *b* shows the absorption of the material obtained when a protagon suspension was added to this crude toxin, spun down, washed by resuspension in water and centrifuging and the adsorbed material dissolved in 0·1 N-NaOH. This curve is typical of a protein (or mixture of proteins) dissolved in alkali, with the absorption maximum shifted to 290 mμ and with an inflexion at about 245 mμ. The absorption curve, *c*, of a purified protein, crystalline trypsin, is shown for comparison. These curves show that when tetanus toxin receptor was added to crude tetanus toxin only protein, and nothing else which absorbed u.v. radiation, was adsorbed. With the amount of protagon used in this experiment it was found that all the toxin, and 22 % of the protein, was adsorbed from the crude toxin preparation. Since 25 % of the protein of the preparation was known to be toxin it seemed that the adsorbed protein consisted only of toxic protein. This was confirmed in the next experiment.

The adsorbed protein consists only of toxic protein

To a series of tubes containing constant amounts of toxin dissolved in constant volumes of 0·133 M-phosphate buffer (pH 7; 60 L+doses/ml.) were added equal volumes of water suspensions containing increasing concentrations of receptor. After standing for 2 hr. at room temperature the tubes were centrifuged, the supernatant fluids set aside, the residues washed, and the adsorbed protein dissolved in 0·1 N-NaOH and estimated. The toxin contents (L+doses/ml.) of the supernatant fluids were determined by assay in mice, and the amount of adsorbed toxin calculated by difference. Figure 2 shows how closely the values for protein adsorbed as determined chemically agreed with the values for toxin adsorbed as determined by biological assay. It is clear that when the receptor was added to the crude tetanus toxin only the toxic protein was adsorbed and the irrelevant proteins unaffected, and that the more receptor was added, the more toxic protein was adsorbed. Each pair of points, for protein and toxin adsorbed, respectively, provides a value for the protein content of one L+ unit of toxin; the mean for the 9 pairs of points in Fig. 2 is 1·91 μg. protein/L+ unit. This figure compares well with the value 2·02–2·15 μg. protein/L+ unit which can be deduced from the data for the pure crystalline toxin of Pillemer *et al.* (1948; 3400–3600 Lf/mg. N; 15·7 % N), if it be assumed that since the crude and crystalline preparations have similar ratios of LD 50/Lf (24,000 and 18,000, respectively) they have the same ratio of Lf/L+ (viz. 1·146; van Heyningen, 1959).

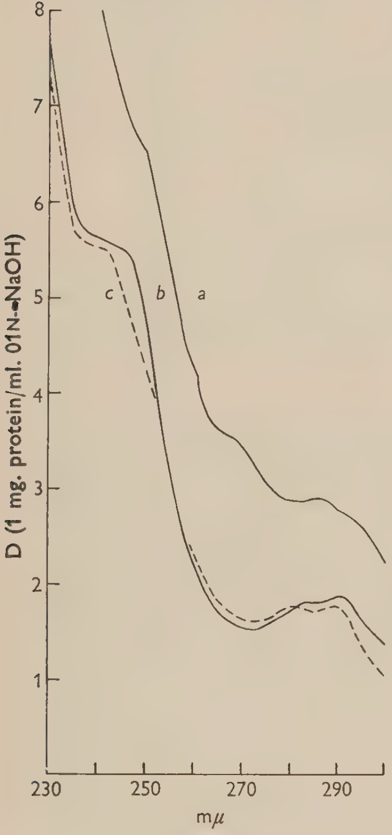

Fig. 1

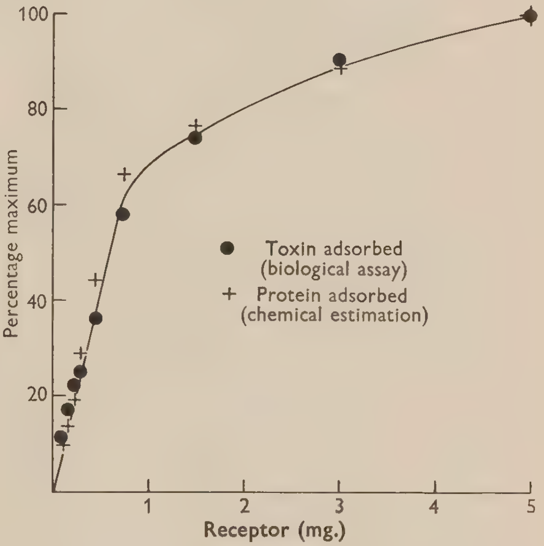

Fig. 2

Fig. 1. Ultraviolet absorption spectra of (*a*) crude tetanus toxin, (*b*) material adsorbed from crude tetanus toxin on to receptor, and (*c*) crystalline trypsin.

Fig. 2. Correlation between protein and toxin adsorbed from crude tetanus toxin on to receptor.

Table 1 shows that when the receptor suspension was added to solutions of a variety of preparations of toxins, enzymes and inert proteins, it adsorbed an appreciable amount of protein only from the tetanus toxin preparations, and to a lesser extent from the tetanus toxoid preparation. The preparations of tetanus toxin XW 1322 and toxoid were comparable since they were both used at a concentration of 120 Lf/ml. and had the same ratio of Lf/mg.

protein. As is known from previous work (Fulthorpe, 1956; van Heyningen, 1959), and as can be seen in Table 1 and in Fig. 4, the amount of tetanus toxin adsorbed increased with increasing concentration of toxin. Simple extrapolation of the data for the two tetanus toxin preparations suggests that if pure toxin had been used about 150 μg. protein would have been adsorbed under the conditions of the experiment.

Table 1. *Adsorption of protein by 250 μg. protagon*

Protein preparation (1000 μg.)	Protein adsorbed (μg.)
Tetanus toxin (crude, XW 1322; 250 μg. toxic protein)	80
Tetanus toxin (less crude, TD 464 D; 750 μg. toxic protein)	130
Tetanus toxoid (crude, *c.* 250 μg. toxoid protein)	21
Diphtheria toxin (crude)	5
Dysentery toxin (crude)	4
Clostridium welchii type A toxin (crude)	4
Cl. welchii type B toxin (crude)	8
Cl. welchii type C toxin (crude)	4
Cl. welchii type D toxin (crude	6
Trypsin (crystalline)	4
Pepsin (crystalline)	3
Lysozyme (crystalline)	14
Serum albumin (purified)	3
Serum α- and β-globulins (purified)	5

The slight adsorption of lysozyme is real, but probably unrelated to the adsorption of tetanus toxin. Figure 3 shows that adsorption of lysozyme took place at low salt concentrations, but diminished sharply as the salt concentration increased, and did not take place in solutions of ionic strength greater than 0·15 (0·067 M-phosphate buffer, pH 7). Similar results were obtained with crystalline trypsin and purified serum albumin, but not with crystalline pepsin, or with the other proteins listed in Table 1. On the other hand, the adsorption of tetanus toxin was independent of the salt content of the milieu.

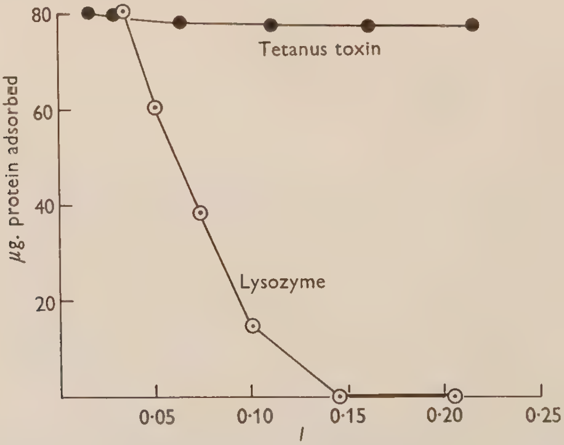

Fig. 3. Effect of ionic strength, *I* (of phosphate buffer, pH 7) on adsorption of tetanus toxin (250 μg.) and lysozyme (250 μg.) by receptor (250 μg.).

Table 2 shows that purified cerebrosides and sphingomyelin had little capacity, as compared with crude protagon, to adsorb protein from the crude toxin preparation. Such capacity as these sphingolipids had to adsorb toxin was probably due to contamination with the receptor substance.

Table 2. *Adsorption of protein from crude tetanus toxin* (1 *mg. protein*) *by various sphingolipids* (250 *µg.*)

Same conditions as Table 1.

Sphingolipid	Protein adsorbed (µg.)
Crude protagon	80
Phrenosine	15
Mixed cerebrosides	15
Sphingomyelin	14

Optimal conditions for adsorption of toxin by receptor

Figure 4 shows the effects of various conditions on the adsorption of toxin from the crude tetanus preparation by protagon. Maximum adsorption was apparently attained within 5–10 min.; but it must be borne in mind that the protagon and the toxin preparation were in contact for a further hour during the subsequent centrifugation. The rapid adsorption of toxin by the protagon suspension contrasts with the slower adsorption by brain (Fulthorpe, 1956; Wassermann & Takaki, 1898). There was little difference between adsorption at 2° and 37°. Adsorption of toxin was maximal at pH values around 7, but precipitation of toxin prevented the determination of adsorption at pH values below 6. Adsorption of toxin increased with increasing toxin concentration, in confirmation of Fulthorpe's (1956) observations with dried brain. The

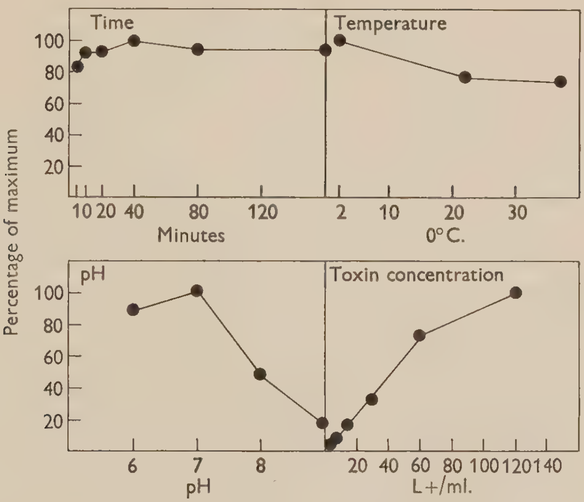

Fig. 4. Effect of various conditions on the activity of the tetanus toxin receptor.

fact that the adsorption of tetanus toxin was independent of salt concentration has already been noted in Fig. 3, and the effect of concentration of receptor has been seen in Fig. 2.

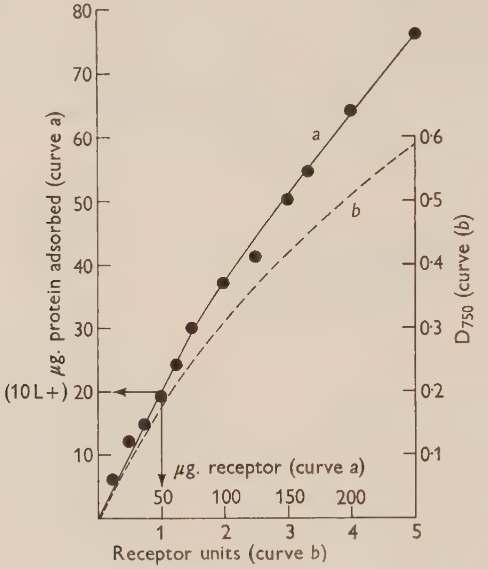

Fig. 5. Activity curve (a) and standard curve (b) derived from it for assay of tetanus toxin receptor.

The activity curve and the standard curve

On the basis of the information conveyed in Figs. 3 and 4 the data for the steep part of the curve in Fig. 2 were redetermined under optimal conditions, and plotted in Fig. 5, curve *a*. It was decided to work at a final toxin concentration of 60 L+/ml., the best compromise between high adsorption and high consumption of toxin. The data were determined as follows. To a series of 1 ml. volumes containing 120 L+ units of toxin (7·68 mg. toxin preparation XW 1322, 1·04 mg. protein)/ml. 0·133 M-phosphate buffer (pH 7) were added equal volumes of aqueous suspensions containing increasing amounts (12·5–250 μg.) of protagon. After standing for 10 min. at room temperature the mixtures were centrifuged, the supernatant fluids gently sucked off, and the gelatinous pellets of residue gently rinsed several times with water. The residues were then suspended in 3 ml. of Reagent C of Lowry *et al.* (1951; 50 ml. 2 % (w/v) Na_2CO_3 in 0·1 N-NaOH + 1 ml. 0·5 % (w/v) $Cu_2SO_4 . 5H_2O$ in 1 % (w/v) sodium tartrate). After 10 min. 0·25 ml. Folin-Ciocalteau reagent (N-acid) was added, and after a further 30 min. the solutions were clarified by centrifugation and the light absorption at 750 mμ determined. From a standard curve obtained with crystalline trypsin treated with the same reagents the amounts of protein adsorbed on the different amounts of protagon, and dissolved by Reagent C, were determined, and a smooth activity curve (Fig. 5*a*) drawn through the points thus obtained. A Receptor Unit (RU) was arbitrarily defined as the amount of receptor that would fix 10 L+ units (i.e. 20 μg. at 2 μg./L+) of toxin (see Discussion, van Heyningen, 1959) and the weight of receptor corresponding to this unit was read off from the activity curve. In the case of the particular preparation of protagon used for this work the Receptor Unit happened to be exactly 50 μg. The smooth curve in Fig. 5*a* was then replotted to give the standard curve in Fig. 5*b* with Receptor Units on the abscissa and colour developed (D_{750}) by the adsorbed protein on the ordinate. The RU by the chemical assay should be smaller than the RU–20 L+ by the biological assay (van Heyningen, 1959) since the chemical assay is determined not only against a smaller amount of toxin, but also in a greater concentration of toxin, in which a greater amount is adsorbed/unit weight receptor.

Table 3. *Reproducibility of chemical assays of receptor activity of a particular preparation of protagon as determined on a number of occasions*

Time after first assay (days)	RU/mg. protagon	Time after first assay (days)	RU/mg. protagon
0	20	30	20
0	20	43	21
0	19	60	19
3	21	61	18
7	20	71	18
15	17	90	19

The procedure for determining the receptor activity (RU/ml.) of an unknown preparation is simple. The weighed sample is suspended in a suitable volume of water in the usual way and a 1 ml. sample of the suspension added to 1 ml. of toxin solution containing 120 L+/ml. 0·133 M-phosphate buffer (pH 7). The procedure described in the previous paragraph is then carried out, except that the value for D_{750} is translated directly into RU by means of the standard curve. A capable technician can easily carry out 20 to 50 assays in 2½–3 hr. Table 3 shows that the assays are reasonably reproducible.

DISCUSSION

The main object of the work reported in this paper was to develop a convenient, quick and reliable method for assaying the receptor; this appears to have been achieved. This work also demonstrates the highly specific affinity of the receptor substance in the grey matter of nervous tissue for tetanus toxin, and thus enhances the significance of the Wassermann–Takaki phenomenon. It increases confidence in the possibility that the receptor might play a part in the action of the toxin, and that combination between these might be the first step leading to the biochemical lesion underlying the pharmacological action of the toxin. The fixation of other proteins, such as lysozyme and trypsin, is probably fortuitous, and irrelevant under physiological conditions of salt concentration. Figure 3 shows that it is abolished in the presence of quite low concentrations of salt, and is therefore likely to be due to electrostatic forces. These forces are unlikely to be responsible for the specific fixation of tetanus toxin, which is unaffected by salt.

The specificity of the receptor for the toxin suggests a number of possibilities which have not yet been explored. Thus it might prove possible to develop a simple means of obtaining highly purified toxin from crude preparations by adsorbing the toxin on protagon, centrifuging and washing the protagon toxin complex, and recovering the toxin from it by dissociation. It should also be possible to assay the toxin, to determine the purity of crude preparations, and to establish whether or not the toxin is identical with a given component of a crude preparation.

The fact that tetanus toxoid retains, although to a much smaller extent, its capacity to be adsorbed by protagon does not necessarily contradict the observations of Pons (1938) and Fulthorpe (1956) that toxoid was not adsorbed by fresh or dried brain, since the receptor activity of protagon is about 40 times as great as that of dried brain. Competitive adsorption of tetanus toxoid is probably responsible for the protective effect of toxoid observed by Lemétayer *et al.* (1950, 1954) and by Raynaud *et al.* (1951) in circumstances where a high concentration of toxoid might compensate for its relatively low affinity for the receptor. They showed that previous injection of massive doses of tetanus toxoid into mice or guinea-pigs delayed or prevented death from the injection 24 hr. later of an otherwise lethal dose of toxin. Raynaud & Wright (1953) reported that the protective effect was shown only by tetanus toxoid and not by diphtheria toxoid. This phenomenon is also no doubt related to Wolters & Fischoeder's (1954) and Fulthorpe's (1956) findings that when brain tissue was first exposed to tetanus toxoid its capacity to absorb tetanus toxin was diminished. However, Fulthorpe showed that diphtheria toxoid also inhibited the capacity of brain to adsorb tetanus toxin.

It was shown in the previous paper (van Heyningen, 1959) that the brain of the highly resistant chicken has the same capacity to fix toxin as the brain of susceptible animals. Evidently the insusceptibility of the chicken is not due to a lack of the receptor in its nervous tissue, but to other causes, which may be connected with the fact that injected toxin disappears rapidly from the circulation of mammals, but not of chickens (see Fildes, 1929). In the present and previous papers it has been shown that the fixation of toxin is unaffected by temperature, which suggests that the fixation of toxin on the receptor is not directly connected with the fact that the toxin is ineffective at lower temperatures, but effective at higher temperatures, in poikilothermic animals, such as the frog (see Fildes, 1929; Wright, 1955).

REFERENCES

FILDES, P. (1929). Medical Research Council: *A System of Bacteriology in Relation to Medicine*, **3**, 298. London: H.M. Stationery Office.

FULTHORPE, A. J. (1956). Adsorption of tetanus toxin by brain tissue. *J. Hyg., Camb.* **54**, 315.

LEMÉTAYER, E., NICOL, L., GIRARD, D., CORVAZIER, R. & CHEYROUX, M. (1950). Recherches expérimentales sur les interactions du serum, l'anatoxine, de la toxine spécifique et des tissus dans le traitement prevéntif et curatif de l'intoxication tétanique. *Ann. Inst. Pasteur*, **79**, 672.

LEMÉTAYER, E., RAYNAUD, M., NICOL, L. & TURPIN, A. (1954). Action préventive spécifique précoce de l'anatoxine tétanique employée à doses massives. *Ann. Inst. Pasteur*, **87**, 1.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

PILLEMER, L., WITTLER, R. G., BURRELL, J. I. & GROSSBERG, D. B. (1948). The immunochemistry of toxins and toxoids. VI. Crystallization and characterization of tetanal toxin. *J. exp. Med.* **88**, 205.

PONS, L. (1938). L'anatoxine tétanique est-elle une toxine ayant perdu son pouvoir de fixation sur le système nerveux? *C.R. Soc. Biol., Paris*, **129**, 209.

RAYNAUD, M., LEMÉTAYER, E., TURPIN, A., NICOL, L. & ROUYER, M. (1951). Installation rapide d'un état de resistance a l'intoxication tétanique expérimentale par l'influence de doses massives d'anatoxine tétanique. *C.R. Acad. Sci., Paris*, **233**, 586.

RAYNAUD, M. & WRIGHT, E. A. (1953). Rapid specific preventive action of tetanus toxoid. *Nature, Lond.* **171**, 797.

VAN HEYNINGEN, W. E. (1959). The fixation of tetanus toxin by nervous tissue. *J. gen. Microbiol.* **20**, 291.

WASSERMANN, A. & TAKAKI, T. (1898). Über tetanusantitoxische Eigenschaften des normalen Centralnervensystems. *Berl. klin. Wschr.* **35**, 5.

WOLTERS, K. L. & FISCHOEDER, E. (1954). Über die Bindung von Tetanustoxin an Hirnsubstanz ohne und nach Vorbehandlung mit Tetanustoxoid. *Z. Hyg. InfektKr.* **139**, 541.

WRIGHT, G. P. (1955). The neurotoxins of Clostridium botulinum and Clostridium tetani. *Pharmacol. Rev.* **7**. 413.

(*Received* 17 *October* 1958)

van Heyningen, W. E. (1959). *J. gen. Microbiol.* **20**, 310–320

Tentative Identification of the Tetanus Toxin Receptor in Nervous Tissue

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SUMMARY: The tetanus toxin receptor in nervous tissue appears to be a ganglioside (mucolipid, strandin, containing: fatty acid, sphingosine, glucose, galactose, acetylgalactosamine, acetylneuraminic acid and amino acid (?) residues). The water-soluble ganglioside occurs in nervous tissue in the form of labile water-insoluble complexes with cerebrosides and sphingomyelins. The solubility of the complexes in water increases with increasing ganglioside content, but decreases when calcium is incorporated. Standing with ganglioside inactivates the toxin.

In a previous paper (van Heyningen, 1959*a*) the specific receptor of tetanus toxin in the grey matter of nervous tissue was shown not to be a cerebroside, as had been thought by earlier workers (Landsteiner & Botteri, 1906; Takaki, 1908), but a closely associated substance. Since attempts to isolate the receptor from crude cerebroside by extraction and adsorption invariably resulted in loss of receptor activity, it was decided to study the separation of the components of the crude material by counter-current distribution between immiscible solvents. As a preliminary to this it was necessary to devise a convenient *in vitro* assay of the receptor (van Heyningen, 1959*b*); this assay proved most useful in the work now reported, which led to the tentative identification of the receptor.

METHODS

The tetanus toxin preparations (XW 1322, TD 464D), the antitoxin and the assay of toxin, the sphingolipid preparations and the method for suspending them in water, the assay of receptor and the method for estimating protein were described in earlier papers (van Heyningen, 1959*a*, *b*). Ganglioside from brain was kindly supplied by Professor E. Klenk of the University of Cologne, and strandin by Dr J. Folch-Pi of Harvard University. Ganglioside was also prepared by the partition-dialysis method of Folch, Arsove & Meath (1951) and by Rosenberg & Chargaff's (1958) modification of this method. Ganglioside was estimated with Bial's reagent by the method of Bohm, Dauber & Baumeister (1954), using Dr Folch-Pi's sample of strandin as a reference standard.

RESULTS

Initial failure to recover receptor in counter-current distribution experiments

The solvent system used in the first counter-current distribution experiment consisted of the two phases obtained when 20 vol. anhydrous methanol, 20 vol. *n*-hexane and 1 vol. water were mixed. When crude protagon was dissolved in equal volumes of the two phases and twenty-four transfers made in the counter-current apparatus, most of the dry weight was recovered in the first few tubes, but very little or no receptor could be recovered in any of the tubes. Similar results were obtained with the solvent system of Gregory & Craig (1948; 15 vol. chloroform, 15 vol. benzene, 23 vol. methanol, 7 vol. water). However, when samples from all the tubes were pooled a high yield of receptor activity was obtained. Evidently the receptor was of complex constitution.

Complex nature of the receptor

Crude protagon was distributed between equal volumes of the two phases of the solvent system of Gregory & Craig (1948), and the solvent evaporated from samples of each phase. The residues were weighed, suspended in water, the suspensions brought to the boil, cooled, and tested for receptor activity. 70 % of the dry weight and only about 8 % of receptor was recovered from the lower phase, and 30 % of the dry weight and no receptor activity at all was recovered from the upper phase. However, when equal volumes of the two phases were mixed, the solvent evaporated off, and the residue suspended in water, an almost complete recovery of receptor activity was obtained. This is shown in Table 1. It appeared therefore that the receptor consisted of at least two components that were practically completely separated by partition between the two phases of the solvent system. That these components of the receptor were moieties of a complex, rather than cofactors, was suggested by the finding that when the material from each phase was independently suspended in water, and the two suspensions mixed, no receptor activity was recovered; but when the two components were added together in solution in organic solvent, the solvent removed and the residue suspended in water, full activity was recovered, as can be seen in Table 2. Apparently it was necessary that the two components should react together in solution in order to reconstitute the receptor. This suggestion was borne out by the fact that the upper phase

Table 1. *Recovery of receptor in two phases of Gregory & Craig* (1948) *solvent system*

RU = Receptor Unit, van Heyningen 1959*b*.

	RU
5 mg. protagon, before adding to 1 ml. lower phase + 1 ml. upper phase	65
3·5 mg. lower phase material	5
1·5 mg. upper phase material	0
3·5 mg. lower phase material + 1·5 mg. upper phase material	56

Table 2. *Upper and lower phase components must react together in solution to reconstitute receptor*

	RU
3·5 mg. lower phase component suspended in water	4
1·5 mg. upper phase component suspended in water	0
Lower phase component in water + upper phase component in water	3
Lower phase component in lower phase solvent + upper phase component in upper phase solvent, solvent removed, residue suspended in water	64

component altered the appearance of the lower phase component. When a suspension of lower phase component was spun down the pellet was white, opaque and slightly granular; but the pellet from a suspension of protagon, or of the reconstituted complex, was translucent and gelatinous.

The lower phase component

Receptor activity was always associated with phrenosine, and the solubility properties of phrenosine were such that it was likely to appear in the lower phase of the Gregory & Craig solvent system. An experiment was therefore done to test whether phrenosine and other cerebrosides could be substituted for the lower phase component. Table 3 shows that phrenosine could indeed be substituted for the lower phase component, but not the upper phase component, and purified mixed cerebrosides (phrenosine, cerasine, nervone) were as effective as phrenosine. In these tests sphingomyelin was apparently ineffective, but later work showed that this conclusion was misleading (see below).

Table 3. *Replacement of lower phase component by phrenosine or mixed cerebrosides*

	RU
3·5 mg. lower phase component	4
1·5 mg. upper phase component	0
2·5 mg. phrenosine	7
2·5 mg. mixed cerebrosides	6
2·5 mg. sphingomyelin	4
Lower phase component + phrenosine	4
Upper phase component + phrenosine	64
Upper phase component + mixed cerebrosides	65
Upper phase component + sphingomyelin	6

In the experiments so far reported the chemical assay for receptor activity (van Heyningen, 1959*b*) was used. Table 4 shows that the results obtained were confirmed with animal experiments. Phrenosine alone and upper phase alone adsorbed little or no protein or toxin from a solution of crude toxin (XW 1322), but the complex of the two components adsorbed 1·94 μg. protein for each L+ unit of toxin. This ratio was in good agreement with the ratio 1·91 previously observed for the adsorption of toxic protein by protagon, and with the ratio of 2·02–2·15 for pure crystalline toxin (see van Heyningen, 1959*b*).

Table 4. *Adsorption of protein and toxin from solutions containing* 30 L+/*ml. by complex of phrenosine and upper phase component*

System	Protein adsorbed (μg.)	L+ toxin adsorbed	Protein per L+ (μg.)
0·5 mg. phrenosine	18	0	—
0·5 mg. upper phase component	0	0	—
0·25 mg. phrenosine + 0·25 mg. upper phase component	155	80	1·94

Formation of water-soluble complexes

Occasionally complexes of phrenosine and upper phase component apparently failed to adsorb tetanus toxin, especially when the upper phase component had been partially purified by extraction with lower phase solvent. Figure 1, curves *a* and *c*, show that this apparent failure to adsorb took place when more than a certain optimum proportion of upper phase component was present in the complex, and that the effect was more marked with partially purified upper phase component. At first it was thought that the effect was due to a water-soluble inhibitor contaminating the upper phase component, and this view appeared to be supported by the finding that there was no decrease of receptor activity with excess upper phase component prepared from an aqueous suspension of protagon that had been dialysed against tap water. However, it was then noticed that the spun-down pellets of receptor complex became progressively smaller as more upper phase component was incorporated, and correspondingly less toxin was carried down. Evidently the complexes became increasingly soluble in water with increasing content of upper phase component, and this was responsible for the apparent failure of the complex to adsorb toxin. Dialysing the protagon against tap water seemed to prevent this solubilization, possibly by introducing calcium into the complex, although partially purified upper phase component was soluble both in water and in 0·05 M-$CaCl_2$ solution. Solutions of partially purified upper phase component in water, and in 0·05 M-$CaCl_2$, were evaporated to dryness, dissolved in upper phase solvent, and added to solutions of phrenosine in lower phase solvent. The solvents were then evaporated off, the residues suspended in water, and the suspensions centrifuged in tared tubes, decanted, dried and weighed. Figure 2 (*a*) shows that complexes containing cerebroside and more than a small proportion

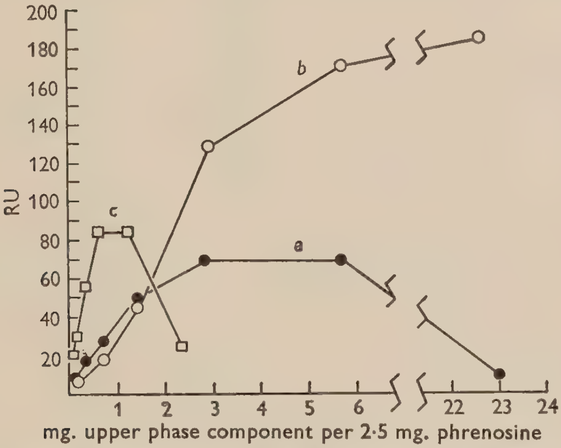

Fig. 1. Effect of varying proportions of upper phase component on activity of receptor complex. (*a*) Crude upper phase component from undialysed protagon. (*b*) Crude upper phase component from dialysed protagon. (*c*) Purified upper phase component from undialysed protagon.

of upper phase component were progressively more soluble in water, but that incorporation of calcium in the complexes prevented them from becoming soluble. Calcium thus affected the solubility of the complex, but not of the upper phase component alone. Figure 2(*b*) shows that similar complexes were formed between upper phase component and sphingomyelin, and that these, with and without calcium, were more soluble than the cerebroside complexes.

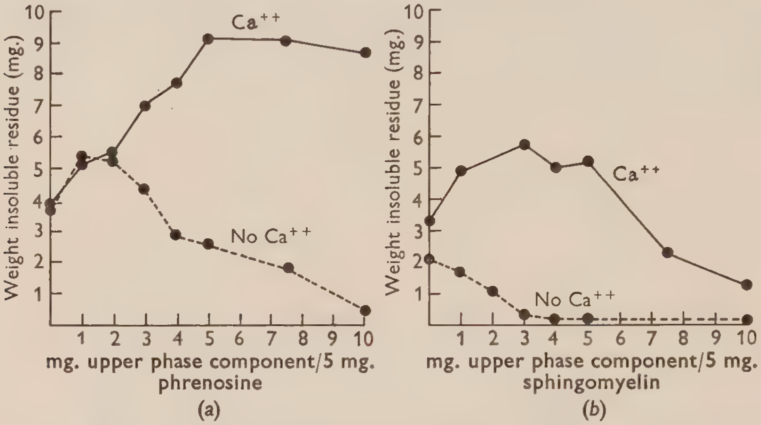

Fig. 2. Effect of varying proportions of upper phase component, and of calcium, on solubility of complexes with (*a*) phrenosine, and (*b*) sphingomyelin.

Since the assay for receptor activity (van Heyningen, 1959*b*) entails spinning out the receptor-toxin complex, it depends not only on the ability of the receptor to combine with toxin, but also on its insolubility in water and its ability to settle in the centrifugal field. Table 5 confirms the effect of calcium in apparently increasing the receptor activity of the complex of upper phase component and phrenosine. It shows further that the complex of upper phase component and sphingomyelin also fixed the toxin. The data in Table 3 had suggested that sphingomyelin could not replace the lower phase component of the receptor, but now it can be seen that this conclusion was erroneously drawn, because the sphingomyelin complex did in fact fix toxin, but, since it could not be spun down, the fixation was undetected.

Table 5. *Effect of incorporation of calcium on ability of receptor complexes to carry down toxin*

	RU	
2·5 mg. upper phase component with:	No calcium	calcium
2·5 phrenosine	39	200
2·5 mg. sphingomyelin	13	133

The upper phase component

The water-insoluble lower phase component of the receptor complex occurs mainly in white matter, whereas the receptor occurs mainly in grey matter (van Heyningen, 1959*a*); it followed therefore that the water-soluble upper phase component should occur mainly in grey matter. The only lipids known to occur in greater concentration in grey matter (0·6–0·7 g./100 g. fresh tissue) than in white matter (0·06–0·07 g./100 g. fresh tissue; LeBaron & Folch, 1957) are the water-soluble gangliosides (see Klenk, 1955), known also as strandin and mucolipids (Folch *et al.* 1951; see also Rosenberg & Chargaff, 1958). The gangliosides probably exist in nature in several forms (see, for example, Svennerholm, 1956; Klenk, 1955). Strandin prepared from grey matter by gentle means (partition dialysis; Folch *et al.* 1951) can be obtained in a homogeneous form as judged by electrophoretic and ultracentrifugal criteria. It is a polymer of high molecular weight (180,000) containing the following residues per molecule: fatty acid (mainly lignoceric), 101; sphingosine, 99; hexose (galactose and glucose in the ratio 8:1), *N*-acetylneuraminic (sialic) acid, 151; *N*-acetylgalactosamine, 36; amino acid, 58 (Rosenberg & Chargaff, 1958). Brain ganglioside has a smaller molecular weight and contains the same residues as the polymer (except the amino acids), but only a fiftieth as many. It is probably derived from the polymer by acid hydrolysis, during the course of which the ratio of *N*-acetylneuraminic acid to sphingosine residues falls from 1·5:1 to 1:1. In aqueous solution ganglioside aggregates to form particles of molecular weight *c.* 250,000.

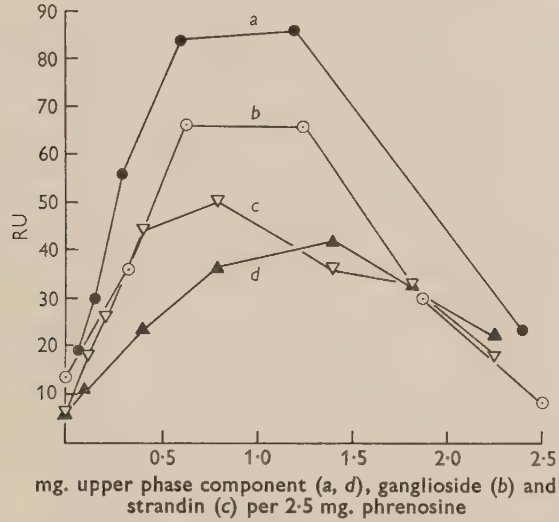

Fig. 3. Substitution of upper phase component (*a*, *d*) by ganglioside (*b*), and strandin (*c*). Different samples of phrenosine used for *a*, *b* and *c*, *d*.

Besides occurring in greater concentration in grey matter than in white, the upper phase component had two further properties in common with gangliosides, viz. (*a*) it was soluble in both water and organic solvents, and (*b*) in the Bial test it yielded a pigment absorbing maximally at 570 mμ. Figure 3 shows that both strandin and ganglioside, tested with different samples of phrenosine, could be substituted for the upper phase component in the receptor complex. The quantitative differences between the curves obtained with the two different samples of phrenosine were probably due to differences in the solubilities of the resultant complexes, or in their ability to settle in the centrifugal field. As with upper phase component, incorporation of calcium prevented complexes containing equal weights of phrenosine and strandin (or ganglioside) from becoming water-soluble. Such complexes contained 40–50 RU/mg., i.e. fixed an equal weight of toxin, since 1 RU fixes 20 μg. toxin (van Heyningen, 1959*b*).

Toxin fixation by ganglioside alone

A ganglioside + cerebroside-Ca complex adsorbing toxin maximally contained roughly equal weights of ganglioside and cerebroside. The fatty acid, sphingosine and hexose residues of ganglioside probably comprise cerebroside moieties within the molecule (see Klenk, 1955; Rosenberg & Chargaff, 1958). This raised the question of the function of the additional cerebroside with which it was necessary to unite the ganglioside in order to constitute the receptor, especially since sphingomyelin could be substituted for cerebroside. It seemed possible that the function of the cerebroside or sphingomyelin component might not be specific with respect to the toxin, but merely to render insoluble the otherwise soluble ganglioside component. The biological and chemical assays (van Heyningen, 1959*a*, *b*) could not be carried out unless the toxin receptor were insoluble in water and therefore removable by centrifugation at 5600 ***g***. The specific affinity of the toxin might be directed to the ganglioside moiety and independent of the cerebroside or sphingomyelin moiety. If the toxin were fixed by the ganglioside alone the resultant complex should be water-soluble, but should have a high molecular weight (> 200,000) and sediment rapidly in the ultracentrifuge. The following experiment was therefore carried out. In each of two Spinco model L ultracentrifuge tubes were placed 3·25 ml. toxin solution containing 40 L+/ml. 0·133 M-phosphate buffer (pH 7). To one tube was added 3·25 ml. water, to the other 3·25 ml. of a solution containing 2 mg. ganglioside/ml. water. The contents of the tubes were mixed and centrifuged at 114,400 ***g*** for 2 hr. At the end of this time the centrifuge was stopped without the brake, the tubes removed without disturbing their contents, and the top 3·25 ml. of each withdrawn and the bottom 3·25 ml. stirred up. The top and bottom halves of the contents of the tubes were then assayed for toxin and, where appropriate, for ganglioside. The results are shown in Table 6. In the control tube containing toxin alone about 40 % of the toxin in the upper half of the tube migrated to the lower half, where it all could be accounted for. In the tube containing ganglioside, less than 15 % of toxin remained in the upper half, but no more toxin was recovered in the lower half than in the control tube. The experiment was repeated several times, always with the same result. The ganglioside thus appeared to carry the toxin down from the upper half of the tube; but the incomplete recovery of toxin was unsatisfactory. It was then found that incomplete recovery of toxin was probably due to inactivation by the ganglioside. Table 7 shows that when toxin was stood for 3 hr. (pH constant at 7) with ganglioside it was 50 % inactivated. The inactivation took time, was greater at 37° than at 1°, and was not increased by increasing the ganglioside concentration from 1 to 5 mg./ml. Ganglioside and strandin were equally effective.

Since the movement of toxin in the ultracentrifugal field could not be followed by measuring the toxin by biological assay, it was decided to follow the movement of protein. For this purpose the toxin preparation TD464D, which contained *c.* 75% toxic protein (van Heyningen, 1959*b*), was used. At the same time parallel measurements were made with crystalline lysozyme and crystalline trypsin, proteins which are adsorbed on the receptor complex at low ionic strength (<0·15; e.g. phosphate buffer (pH 7) <0·067 M; van

Table 6. *Effect of ganglioside on ultracentrifugation of tetanus toxin*

Recovery of toxin in upper and lower halves of centrifuge tubes after centrifugation for 2 hr. at 114,400 *g*.

	% Recovery			
	Ganglioside		Toxin	
System	Upper half	Lower half	Upper half	Lower half
Toxin + water	—	—	30	72
Toxin + ganglioside	8·3	85·0	<7·5	72

Table 7. *Inactivation of tetanus toxin by ganglioside*

Conc. ganglioside (mg./ml.)	Time (min.)	Temp. (°C)	Inactivation of toxin (%)
2	10	15	0
1	180	37	50
5	180	37	50
5	180	1	12
5	180	37	50
0	180	37	0

Table 8. *Effect of ganglioside on ultracentrifugation of tetanus toxin (c. 75% pure) and pure lysozyme and trypsin*

Recovery of protein and ganglioside in upper and lower halves of centrifuge tubes after centrifugation for 2 hr. at 114,400 *g*.

		% Recovery			
		Ganglioside		Protein	
System	Ionic strength	Upper half	Lower half	Upper half	Lower half
Toxin + water	0·15	—	—	35·6	68·8
Toxin + ganglioside	0·15	8·2	94·1	9·9	94·6
	0·30	8·8	94·1	9·9	86·2
Lysozyme + water	0·15	—	—	34·8	53·2
Lysozyme + ganglioside	0·15	9·2	82·1	29·3	69·2
	0·30	9·5	95·0	37·8	67·1
Trypsin + water	0·15	—	—	38·4	62·0
Trypsin + ganglioside	0·15	9·4	87·2	35·9	67·1
	0·30	8·0	95·0	37·8	62·1

Heyningen, 1959*b*). Similar experiments were set up as those reported in Table 6, except that the protein in the upper and lower halves of the contents of each centrifuge tube was determined, rather than the L+ units (Table 8). In a centrifugal field of 114,400 **g** about 80 % of the ganglioside in the upper half migrated to the lower half of the tube, and when toxin was mixed with ganglioside about 80 % of the protein migrated, whereas only 29 % of the protein migrated in the control tube containing toxin alone ganglioside had no effect on the migration of trypsin, and only a slight effect on the migration of lysozyme, which was abolished when the salt concentration was doubled. Evidently it is the ganglioside component which specifically fixes the toxin.

DISCUSSION

The tetanus toxin receptor in nervous tissue can be identified, tentatively at any rate, as a ganglioside. The ganglioside is water-soluble, but in nervous tissue it apparently exists in the form of insoluble complexes with water-insoluble cerebrosides and sphingomyelins (and possibly with other substances as well). It is possible that the receptor is not a ganglioside itself, but a contaminant accompanying it and resembling it in its solubility properties. If this were the case the contaminant would have a very high capacity to fix toxin since the highly purified strandin preparation provided by Dr Folch-Pi fixed double its weight of toxin under the conditions of testing. The constitution of the gangliosides has not yet been precisely defined, and is unlikely to be defined for some time. They form particles of high molecular weight, containing nearly 700 residues of six different kinds, and probably exist in nature in several different forms. The mucolipids of Rosenberg & Chargaff (1958) differ from ganglioside in the important respect that they contain one more *N*-acetylneuraminic acid residue for every two sphingosine residues, and it appears that this residue, but not the other two *N*-acetylneuraminic acid residues, may be split off by the action of neuraminidase. This is suggested by the finding of Rosenberg, Howe & Chargaff (1956) that ganglioside did not inhibit influenza virus haemagglutination and was not attacked by neuraminidase, whereas a high-molecular mucolipid preparation did inhibit haemagglutination, and nearly a third of its *N*-acetylneuraminic acid was split off by neuraminidase. Tetanus toxin, however, does not distinguish between ganglioside and strandin, and fixation of the toxin is therefore apparently independent of the postulated enzymically-removable *N*-acetylneuraminic acid residue. It will be interesting to determine what is the smallest fragment of ganglioside that can still fix toxin.

The fact that tetanus toxin is slowly inactivated, as well as fixed, by ganglioside (or a contaminant) is interesting and probably important. This phenomenon has not yet been investigated in detail and further studies are in progress.

The formation of complexes between different brain lipids, held together by bonds weaker than covalent bonds, is to be expected (see LeBaron & Folch, 1957), and the insoluble component of the ganglioside + cerebroside/sphingomyelin complex no doubt has an important biological function. This work shows that the formation of such complexes can lead to the insolubilization of soluble ganglioside, or alternatively, to the solubilization of insoluble cerebrosides and sphingomyelin.

The experiments on the interaction of the receptor and the toxin were done at toxin concentrations far greater than would be encountered in the course of natural infection. The total weight of ganglioside in the nervous tissue of a mouse (say 1–5 mg.?) is many million times that of a lethal dose of toxin (10^{-7} mg.), but at the concentration of toxin in a mouse dying of a single lethal dose a much greater amount of ganglioside would probably be needed to fix that dose than would be necessary under the conditions of the receptor assay (at 800,000 LD_{50}/ml. protagon fixes 50,000 times as much toxin as at 20 LD_{50}/ml. [van Heyningen, 1959*a*]). Moreover, only a small proportion of the total ganglioside, e.g. part of that situated in the anterior horn cells of the spinal cord, need be involved in a fatal outcome.

What the significance is of the fixation of tetanus toxin by the receptor in nervous tissue is not yet clear. Since the discovery of the Wassermann-Takaki phenomenon sixty years ago it has been thought that the fixation of toxin was directly connected with its mode of action (e.g. see Fildes, 1929). The specificity (van Heyningen, 1959*b*) and affinity of the receptor for the toxin, the fact that the inhibitory synapses and receptor are both in grey matter, and the fact that large doses of toxoid inhibit fixation of the toxin as well as its lethal action (see Discussion, van Heyningen, 1959*b*), all support this idea. It is possible that the ganglioside is the susceptible substance of the toxin, and that combination between the toxin and the ganglioside may be the first step leading to the biochemical lesion underlying the characteristic pharmacological action of the toxin. Another possibility is that the receptor may be responsible for the adsorption of the toxin from the site of infection and its centripetal transport in the nerve trunks to its site of action in the spinal cord and brain, and for its selective concentration and localization within nerve cells. Recent evidence suggests that cerebrosides are situated mainly within the myelin sheaths and membranes of nerve cells and their fibres, whereas gangliosides are situated within the nerve cell bodies and their dendrites and axons (see Svennerholm, 1957). Although the overall concentration of ganglioside in nerve trunks is very small (Folch-Pi & LeBaron, 1957), and the capacity of nerve trunks to fix toxin low compared with that of brain (Fulthorpe, 1956), the significant concentration of ganglioside may be within the axon. On the other hand, it has been suggested that the transport of toxin takes place in the space between the nerve fibres, not in the nerve fibres themselves, and to be actuated by pressure following movement of limbs (see Wright, 1955). The obvious question, whether the ganglioside undergoes any change after fixing the toxin, is at present being investigated.

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REFERENCES

Bohm, P., Dauber, S. & Baumeister, L. (1954). Über Neuraminsäure, ihr Vorkommen und ihre Bestimmung in Serum. *Klin. Wschr.* **32**, 289.

Fildes, P. (1929). In Medical Research Council: *A system of Bacteriology in Relation to Medicine*, **3**, 298. London: H.M. Stationery Office.

Folch, J., Arsove, S. & Meath, J. A. (1951). Isolation of brain strandin, a new type of large molecule tissue component. *J. biol. Chem.* **191**, 819.

Folch-Pi, J. & LeBaron, F. N. (1957). Chemical composition of the mammalian nervous system. In *Metabolism of the Nervous System*, p. 67. Ed. D. Richter. London: Pergamon Press.

Fulthorpe, A. J. (1956). Adsorption of tetanus toxin by brain tissue. *J. Hyg., Camb.* **54**, 315.

Gregory, J. D. & Craig, L. C. (1948). Counter-current distribution of gramicidin. *J. biol. Chem.* **172**, 839.

Klenk, E. (1955). The pathological chemistry of the developing brain. In *Biochemistry of the Developing Nervous System*, p. 397. New York: Academic Press Inc.

Landsteiner, K. & Botteri, A. (1906). Über Verbindungen von Tetanustoxin mit Lipoiden. IV. *Zbl. Bakt.* (*Orig.*), **42**, 562.

LeBaron, F. N. & Folch, J. (1957). Structure of brain tissue lipids. *Physiol. Rev.* **37**, 539.

Rosenberg, A. & Chargaff, E. (1958). A study of a mucolipide from ox brain. *J. biol. Chem.* **232**, 1031.

Rosenberg, A., Howe, C. & Chargaff, E. (1956). Inhibition of influenza virus haemagglutination by a brain lipid fraction. *Nature, Lond.* **177**, 234.

Svennerholm, L. (1956). Composition of gangliosides from human brain. *Nature, Lond.* **177**, 524.

Svennerholm, L. (1957). Qnantitative estimation of gangliosides in senile human brains. *Acta Soc. Med. Uppsala*, **62**, 1.

Takaki, T. (1908). Über Tetanusgift bindende Bestandteile des Gehirns. *Beitr. chem. Physiol. Path.* **11**, 288.

van Heyningen, W. E. (1959*a*). The fixation of tetanus toxin by nervous tissue. *J. gen. Microbiol.* **20**, 291.

van Heyningen, W. E. (1959*b*). Chemical assay of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.* **20**, 301.

Wright, G. P. (1955). The neurotoxins of Clostridium botulinum and Clostridium tetani. *Pharmacol. Rev.* **7**, 413.

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Osmotic Properties of Protoplasts of *Micrococcus lysodeikticus*

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SUMMARY: Protoplasts were released from *Micrococcus lysodeikticus* by the action of lysozyme in media containing sucrose, NaCl or mixtures of these. Empirical corrections by two methods were made for the effect of refractive index on the optical density of the protoplast suspensions measured at 500 mμ. With the assumption, valid for some similar systems, that the optical density of a protoplast suspension is inversely proportional to the protoplast volume, the protoplasts were shown to behave as osmometers at osmotic pressures above the threshold for lysis and to follow a van't Hoff–Boyle law. The osmotically-inactive volume in a standard stabilizing medium containing M-sucrose + 0·05 M-NaCl (osmotic pressure 37·8 atm.) was *c.* 76 %. Protoplasts formed in hypotonic media were more resistant to osmotic explosion than those formed at higher medium concentrations and subsequently diluted.

A cell which lacks structural rigidity will respond to changes in the osmotic pressure of its environment in a manner determined by the selective permeability properties of the external membrane. When the membrane is impermeable to an extracellular solute but freely permeable to the solvent the volume changes produced by osmosis in a system exposed to media of different osmotic pressure should follow the van't Hoff–Boyle law:

$$\pi(V - V_0) = \text{constant}, \tag{1}$$

where π is the osmotic pressure of the medium, V is the volume of the cell and V_0 is that volume of the cell which does not respond to osmotic influences. This law is followed by the cells of many higher organisms (Lucke, 1940). If, as suggested by recent work (McQuillen, 1955; Mitchell & Moyle, 1956*a*, *b*; Gilby, 1957), the protoplasts of *Micrococcus lysodeikticus* are surrounded by an osmotic membrane of low mechanical strength, these too should behave similarly.

Protoplasts of *Bacillus anthracis* and *B. megaterium* have been studied previously by using measurements based on microscopy. Stähelin (1954) investigated *B. anthracis* in sucrose and in NaCl solutions by qualitative methods. Weibull (1955) showed that protoplasts of *B. megaterium* followed equation (1) in sucrose, glucose and polyethylene glycol, but not in urethane. In the present investigation it will be shown that protoplasts of *Micrococcus lysodeikticus* also behave as osmometers in sucrose and NaCl solutions. The method evolved uses the optical properties of the protoplast suspensions and avoids some of the experimental difficulties of direct measurement. However, in its present development it does not permit the calculation of absolute volumes.

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METHODS

Organism used. Micrococcus lysodeikticus (NCTC 2665) was grown on a beef tryptic digest agar medium at pH 7·2 in 20 oz. bottles. Each bottle was inoculated with *c.* 20 ml. of a 48 hr. broth culture and the organisms harvested in distilled water after *c.* 20 hr. incubation at 37°. After passage through a coarse glass filter, the bacteria were washed three times with distilled water by centrifugation.

Protoplast formation. Protoplasts were prepared from *Micrococcus lysodeikticus* by treatment with lysozyme in either of two ways. In some experiments, the organisms were suspended at a concentration equiv. to 0·5 mg. dry wt./ml. in either NaCl up to 2 M or sucrose up to 2 M containing 0·05 M-NaCl, and then treated with lysozyme, 10 μg./ml. for 30–60 min. In other cases, protoplasts were prepared by reacting the organisms at equiv. 10 mg. dry wt./ml. in M-sucrose + 0·05 M-NaCl, with lysozyme 100 μg./ml. for 30 min. These suspensions were subsequently diluted to be equiv. 0·5 mg. dry wt. organism/ml. in media which yielded final compositions of either 0·05 M-NaCl with various concentrations of sucrose, 0·05 M-sucrose with various concentrations of NaCl, or 0·5 M-sucrose with various concentrations of NaCl. Protoplast membranes were prepared by the method previously described (Gilby, Few & McQuillen, 1958) and suspended at equiv. 1 mg. membrane dry wt./ml. in NaCl solutions up to 2 M.

Spectrophotometric measurements. The optical densities of the protoplast and membrane suspensions were measured at 500 mμ with a Unicam SP 600 spectrophotometer.

RESULTS

The values of the optical density, measured at 500 mμ, of the various protoplast suspensions are plotted in Fig. 1 as a function of the osmotic pressure of the medium. The osmotic pressure of the standard stabilizing medium 1 M-sucrose + 0·05 M-NaCl used in previous work (Gilby & Few, 1957*a*, *b*) is 37·8 atm. The results confirm that this is above the threshold for osmotic lysis. The sigmoid shape of the curves in the region of rapid change is a reflexion of the distribution of stability and of osmotic properties in the protoplast population, evident also in the size variation of protoplasts observed at fixed osmotic pressure in phase-contrast micrographs (Gilby, 1957). In contrast, both protoplast 'ghosts' and intact bacteria are more uniform in size. At osmotic pressures where the protoplasts are stable, the optical density of the suspensions, with the exception of those in pure sucrose, increases as the osmotic pressure increases. This is attributed to a decrease in volume of protoplasts. It is not known whether crenation occurs. With sucrose solutions, the increase in refractive index masks this effect. The differences between the curves at higher osmotic pressures are due to refractive index effects.

In order to use the present results to determine whether *Micrococcus lysodeikticus* protoplasts follow equation (1), it is necessary both to eliminate the effect on the optical density of the different refractive indices of the media and to relate the optical density to the average volume of the protoplasts.

There is no theory of light scattering applicable to particles the size of protoplasts (0·5–1 μ); hence it is necessary to rely on empirical methods.

Corrections for refractive index of medium. Two methods were used.

Method A. From the known composition of each medium its refractive index was ascertained from the literature and, in many cases, checked by measurement with an Abbé refractometer. The results in Fig. 1 were then used to plot, at fixed values of osmotic pressure, the optical density as a function of Δn, the difference in refractive index of the medium and water. A smooth curve was drawn through the points and extrapolated to estimate the optical density in a medium of refractive index equal to water.

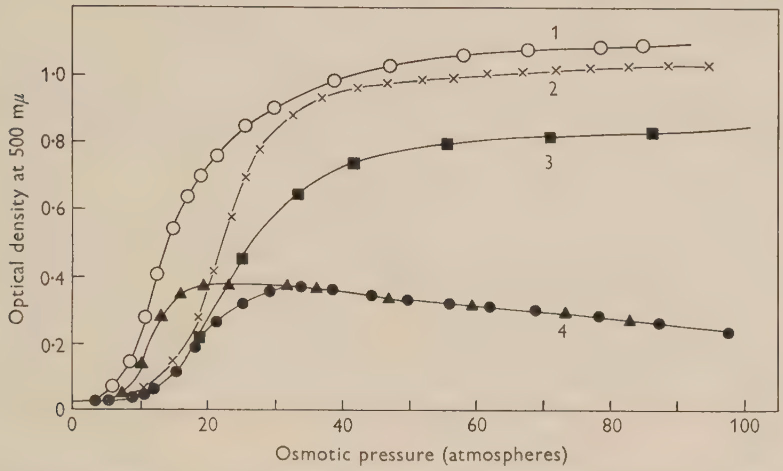

Fig. 1. Optical density of protoplasts of *Micrococcus lysodeikticus* suspended in solutions of different osmotic pressure. Protoplast suspensions at equiv. 0·5 mg. dry wt./ml. in (1) NaCl, (2) NaCl + 0·05 M-sucrose, (3) NaCl + 0·5 M-sucrose, (4) sucrose + 0·05 M-NaCl. ▲, Protoplasts formed in test medium; ●, protoplasts preformed in M-sucrose + 0·05 M-NaCl and transferred to test medium.

Method B. This was based on the results in sodium chloride alone. Figure 2 shows the optical density of protoplast membranes suspended in solutions of NaCl. At the lowest concentrations the membranes are unstable (Gilby *et al.* 1958). The curve in the stable region was therefore extrapolated to estimate the optical density of the membranes in water. It has been assumed that the different optical densities in Fig. 2 arise from the effect of the refractive index of the medium and that, in this regard, protoplast membranes and protoplasts behave similarly. At each concentration (osmotic pressure) of NaCl, the ratio for membranes of the optical density in the medium to that in water was applied to correct the results with protoplasts to correspond to a medium of the refractive index of water.

Application of method A gave curve (1) of Fig. 3; results with Method B were so similar that a separate plot is not warranted.

Quantitative test of behaviour of protoplasts as osmometers. In the treatment of the results which follows, it is assumed that the optical density of a protoplast suspension is inversely proportional to the average protoplast volume.

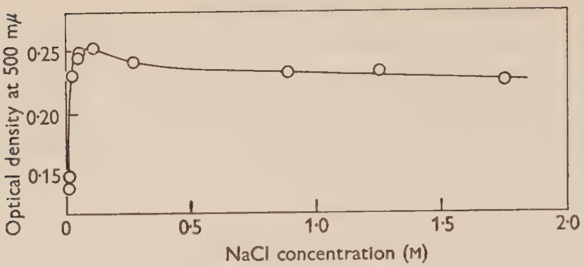

Fig. 2. Optical density of protoplast membranes of *Micrococcus lysodeikticus* suspended in NaCl. Protoplast membranes at equiv. 1 mg. dry wt. membrane/ml.

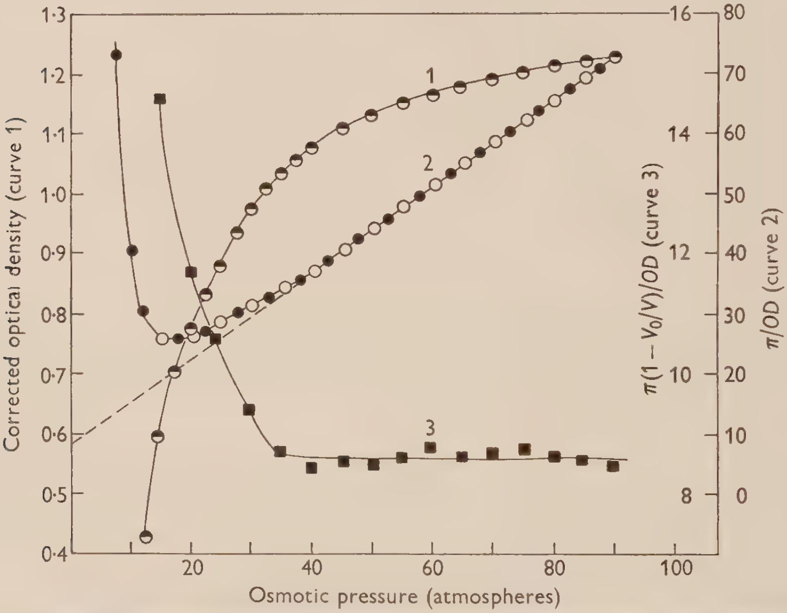

Fig. 3. Optical properties of protoplasts of *Micrococcus lysodeikticus* as a function of osmotic pressure. (1) Optical density at 500 mμ corrected for refractive index of medium (method A). (2) Test of equation (3). Plot of π/OD against π. ●, Refractive index corrections by method A; ○, refractive index corrections by method B. (3) Test of equation (4). Plot of $\frac{\pi}{OD}\left(1-\frac{V_0}{K}OD\right)$ against π.

Equation (1) may be written:

$$\pi(V-V_0) = C, \tag{2}$$

where V and V_0 now refer to the volume of protoplasts in a suspension.

If $V = K/OD$ where K is a constant and OD is the optical density of a suspension at 500 mμ

then
$$\frac{\pi}{OD} = \pi\frac{V_0}{K}+\frac{C}{K}. \tag{3}$$

Alternatively, equation (2) may be written

$$\pi\left(1-\frac{V_0}{V}\right) = \frac{C}{V},$$

therefore
$$\pi\left(1-V_0\frac{OD}{K}\right)=C\frac{OD}{K},$$

and
$$\frac{\pi}{OD}\left(1-\frac{V_0}{K}OD\right)=\frac{C}{K}. \tag{4}$$

From equation (3) it follows that, provided the protoplasts follow a van't Hoff–Boyle Law expressed by equation (1) and the assumptions detailed are valid, a plot of π/OD against π should be linear. Such a plot is shown in curve (2) of Fig. 3. The two symbols refer to calculations using method A or method B to correct for refractive index and these indicate perfect agreement. It is apparent that the results fit equation (3) for osmotic pressures greater than 30 atm. At lower osmotic pressures the results deviate because of lysis of protoplasts. The slope of the linear portion is equal to V_0/K.

The value of V_0/K obtained from the plot of equation (3) can then be inserted in equation (4). This has been done in curve (3) of Fig. 3 and the values of $\frac{\pi}{OD}\left(1-\frac{V_0}{K}OD\right)$ plotted against π. In conformity with equation (4), the values are constant at osmotic pressures greater than 30 atm., the same range of validity as equation (3) from which V_0/K was derived.

DISCUSSION

The experimental methods described possess some advantages, particularly in convenience and the use of a large population, over the method of direct measurement by microscope as used, for example, by Weibull (1955). However, the treatment of results depends on the assumption that the optical density of a protoplast suspension is inversely proportional to the protoplast volume. Because of their small size and fragility the experimental confirmation of this by methods such as haematocrit measurements is likely to prove difficult. Comparable information on other similar systems is rare. With erythrocytes it is sometimes found that the relative light transmission of a system decreases linearly as the cell volume increases. Over a wide range of optical densities, log I_0/I will then be inversely proportional to volume. Further, Wilbur & Collier (1943) found that the product of the optical density and the volume of erythrocytes was constant over a wide range of NaCl concentrations. With rat liver mitochondria also, it was established empirically (Tedeschi & Harris, 1955, 1958) that the optical density of a suspension was inversely proportional to the mitochondrial volume. Although not quantitatively applicable to the present case because the particle size is too great and refraction may occur, Mie theory does predict that as the particle size becomes larger the optical density of a suspension can, under certain conditions, decrease (Heller & Pangonis, 1954). Nevertheless, in the present case the final justification for the assumption lies in the low probability of obtaining the curves (2) and (3) in Fig. 3 in the event of the relation not holding.

The results outlined therefore support the conclusion that protoplasts of *Micrococcus lysodeikticus* behave as osmometers at osmotic pressures above the threshold for lysis. It follows that they must be readily permeable to water. In a medium of low osmotic pressure, the protoplasts imbibe water and, lacking mechanical rigidity and the restraint of the cell wall, they rupture. Since $(V_0/K)\,OD = V_0/V$, then the calculation of V_0/K from equation (3) and Fig. 3, curve (2), as set out in the previous section, also enables the calculation of the ratio V_0/V at any osmotic pressure for which the value of the optical density corrected for refractive index is known. For example, in the standard stabilizing medium of M-sucrose + 0·05 M-NaCl, the osmotically inactive volume is approximately 76 % of the total protoplast volume. Until the value of K is determined, and the basic assumption on the relation between volume and optical density thereby tested independently, it is not possible to give other than relative figures.

Weibull (1955) noted that although protoplasts of *Bacillus megaterium* followed equation (1) after formation, the size of freshly prepared protoplasts was independent of the osmotic pressure of the medium in which they were formed. This does not appear to be so with protoplasts of *Micrococcus lysodeikticus* at osmotic pressures above the stabilization threshold. Furthermore, the optical density of a suspension of *M. lysodeikticus* protoplasts in a medium of given composition is the same (Fig. 1) whether the protoplasts have been formed in that medium or else have been prepared in the standard 1 M-sucrose medium and subsequently transferred to the test medium. However, at osmotic pressures below 30 atm., there is evidence that the protoplasts in this substabilization range were more stable when formed in the medium than when transferred from M-sucrose. Apparently the protoplasts are more sensitive to a suddenly applied osmotic shock, or else may be able to adjust to their environment while losing their cell walls. These observations were not due to momentary exposure to grossly hypotonic solutions; this was rigorously avoided when changes in medium were being effected.

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REFERENCES

GILBY, A. R. (1957). *The Physical Chemistry of Bacterial Protoplasts.* Ph.D. Dissertation, University of Cambridge.

GILBY, A. R. & FEW, A. V. (1957*a*). The reactivity of ionic detergents with *Micrococcus lysodeikticus. Nature, Lond.* 179, 422.

GILBY, A. R. & FEW, A. V. (1957*b*). Surface chemical studies on the protoplast membrane of *Micrococcus lysodeikticus. Proc. 2nd Int. Congr. Surface Activity,* 4, 262. London: Butterworths Scientific Publishers.

GILBY, A. R., FEW, A. V. & MCQUILLEN, K. (1958). The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus. Biochim. biophys. Acta,* 29, 21.

HELLER, W. & PANGONIS, W. J. (1954). Light scattering of spherical colloidal particles. *J. chem. Phys.* 22, 948.

LUCKE, B. (1940). The living cell as an osmotic system and its permeability to water. *Cold Spr. Harb. Symp. quant. Biol.* 8, 123.

McQuillen, K. (1955). Bacterial protoplasts, I. Protein and nucleic acid metabolism in protoplasts of *Bacillus megaterium*. *Biochim. biophys. Acta*, **17**, 382.

Mitchell, P. & Moyle, J. (1956*a*). Osmotic function and structure in bacteria. In *Bacterial Anatomy*. *Symp. Soc. gen. Microbiol.* **6**, 150.

Mitchell, P. & Moyle, J. (1956*b*). Liberation and osmotic properties of the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea*. *J. gen. Microbiol.* **15**, 512.

Stähelin, H. (1954). Über osmotisches Verhalten und Fusion nackter Protoplasten von *Bacillus anthracis*. *Schweiz. Z. allg. Path.* **17**, 296.

Tedeschi, H. & Harris, D. L. (1955). The osmotic behaviour and permeability to non-electrolytes of mitochondria. *Arch. Biochem. Biophys.* **58**, 52.

Tedeschi, H. & Harris, D. L. (1958). Some observations on the photometric estimation of mitochondrial volume. *Biochim. biophys. Acta*, **28**, 392.

Weibull, C. (1955). Osmotic properties of protoplasts of *Bacillus megaterium*. *Exp. cell. Res.* **9**, 294.

Wilbur, K. M. & Collier, H. B. (1943). A comparison of the haemolytic actions of lysolecithin and saponin. *J. cell. comp. Physiol.* **22**, 233.

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Experiments on Splash Dispersal of Fungus Spores

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SUMMARY: Splash dispersal (first studied by Faulwetter, 1917*a*, *b*) is characteristic of many bacterial plant pathogens and slime-spored fungi. The mechanism of splash has been studied in the laboratory under simplified conditions with water drops falling from known heights on to thin films of a suspension of conidia of *Fusarium solani* spread on horizontal glass surfaces. The resulting splash droplets were caught, counted and measured by the naphthol green B slide method (Liddell & Wootten, 1957). Both the total number of droplets produced and of those carrying spores increased as the film thickness decreased, and as the size and velocity of the incident drop increased.

One incident drop 5 mm. in diameter falling on a horizontal film of spore suspension 0·1 mm. thick on a glass surface produced over 5200 splash droplets of which over 2000 carried one or more spores. The sizes of splash droplets ranged from 5 μ to about 2400 μ, and their size distribution was of the log probability type. The median diameter was 70 μ for all droplets, and 140 μ for droplets carrying spores. The medium horizontal distances travelled by these droplets in still air was 10 and 20 cm. respectively. Increasing the film thickness to 0·5 and 1·0 mm. reduced the total resulting droplets to 3500 and 2100 (1600 and 500 carrying spores), respectively. Neither median diameter nor median distance of horizontal travel were much altered by varying the diameter of incident drop or film thickness. Droplets of diameters between 164 and 655 μ tended to travel further than either smaller or larger droplets. Small spores such as those of *Gloeosporium album*, *Colletotrichum lindemuthianum* and *Nectria cinnabarina* were even more readily picked up from suspension than the macroconidia of *Fusarium solani*.

The number of droplets deposited per unit area on a horizontal plane decreased rapidly with increasing distance from point of impact, and in still air few droplets travelled beyond 70 cm.

Splash on a twig bearing conidial fructifications of *Nectria cinnabarina* produced 2000 spore-carrying droplets, and the sequence of events is shown by high-speed photography.

A splash droplet consists of an intimate mixture of liquid from both the incident drop and the surface film. The larger splash droplets contain spores if either the incident drop or the surface film is a spore suspension. Both raindrops falling at terminal velocity and drops falling more slowly from vegetation may operate the splash dispersal mechanism. Splash in rain or drip from trees may act as a complete dispersal mechanism in still air, or as a 'take-off' mechanism leading to dispersal by wind. Both functions are important in plant epidemiology.

Many fungi have mechanisms liberating their spores into dry air and their dispersal has been studied extensively. Many others produce their spores embedded in mucilage, which, as shown by Stepanov (1935), prevents their removal by wind. Field workers recognize that some of these slime-spored fungi are spread by insects while others may be spread by the action of rain and wind together. Faulwetter (1917*a*, *b*) splashed dyes on white paper, and acetic acid on litmus paper, in seeking an explanation of dispersal of bacterial crop-pathogens by the action of rain splash and wind. Little is known about spore dispersal by rain splash, in spite of the part it apparently plays in many economically important crop diseases, such as *Gloeosporium* rot of apples, black-arm (*Xanthomonas malvacearum*) of cotton, dry rot (*Diplodia zeae*) of maize, and celery leaf spot (*Septoria* spp.).

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Raindrops vary in size from 0·2 to about 5·0 mm. diam. (drops larger than this are unstable and break up before reaching terminal velocity), and they fall at terminal velocities varying from 1·0 m./sec. for drops 1 mm. diam. to 7·8 m./sec. for drops 5 mm. diam. Small raindrops normally greatly outnumber larger ones, but rain from convective or thunder clouds has relatively more large drops than have frontal or orographic rain (Mason, 1957).

Knowledge of the events during a splash comes from studies of water drops in the laboratory by Worthington & Cole (1897) and Edgerton & Killian (1939); and from a theoretical and experimental study by Engel (1955). But most of these studies are inapplicable in detail as they deal with splashes from drops falling on deep layers of liquid or on dry surfaces. Splash on a deep layer usually produces a bubble (Blanchard & Woodcock, 1957). In splash from a thin film bubbles are seldom observed and the sequence of events is illustrated by Edgerton's beautiful photograph which forms the frontispiece to the new edition of d'Arcy Thompson's *Growth and Form*.

Data from all these sources suggest the following sequence of events. When a raindrop falls on a horizontal surface covered with a thin film of water a crater-shaped wall of liquid is formed. The wall of the crater moves outwards and upwards, the rim breaks up under surface tension into rays, and these rays in turn break up into chains of minute droplets which are shot from the ends of the rays into the air in an upward and outward direction. The trajectory of these droplets is determined by gravity and friction between the minute droplets and the air. Ultimately the droplets will be deposited on a surface or they will evaporate.

The sequence of events when micro-organisms are dispersed by rain splash is envisaged as follows. Dry spores are held firmly to their substratum by the mucilage in which they are embedded. When rain comes the first drops merely wet the surface, swelling the mucilage and allowing the spores or bacteria to float in a film of water (Nisikado, Inouye & Okamoto, 1955). From this film they are removed in the splash droplets thrown into the air by impact when succeeding raindrops strike the surface film. The object of our work was to estimate the number and spore-carrying power of droplets produced by splash under various conditions.

METHODS

Drops of tap-water (*incident drops*) were allowed to fall vertically from known heights in still air in the laboratory on to thin layers of spore suspension spread on horizontal glass slides (*target films*). The droplets thrown up by the splash (*splash droplets*) were sampled to determine their numbers, size, distance of travel in still air, and whether or not they carried spores. The splash pattern was reconstructed from samples of the resulting droplets collected on 3 × 1 in. microscope slides placed horizontally on the same level as the target film of spore suspension and at various distances. Preliminary tests by Guthrie (1957) on methods of studying the droplets resulting from a splash led to the adoption of the naphthol green B slide detection method developed at Porton by Liddell & Wootten (1957) for determining droplet size distributions in fog by using a Cascade Impactor. Droplets are caught on microscope slides coated with a thin layer of gelatine dyed with naphthol green B. Each droplet leaves a circular 'stain' with a clear centre and defined dark green margin that can easily be measured under a low-power microscope. The relation between stain diameter and droplet diameter is determined by calibration with droplets of known diameter. Preliminary tests gave reproducible results when an average of five or ten splashes was taken. The methods used in the main series of experiments were modified as required for individual tests as described in later sections.

Production of incident drops. In imitation of raindrops, tap water was dropped from the end of vertical pipettes held at the required height near the ceiling of the laboratory or hall in a tower constructed from 'Dexion'. The drop sizes used were approximately 2·0, 3·0, 4·0 and 5·0 mm. in diameter. The end of the pipette was ground smooth and coated with silicone grease. Its outside diameter was 2·0 mm. for 4·0 mm. diam. drops, and 4·5 mm. for 5·0 mm. drops. For smaller drops glass capillaries were drawn out to 0·2 mm. outside diam. for 2 mm. drops or slightly larger for 3·0 mm. drops and were allowed to drip continuously under a constant head of water. Except for the largest drops from the greatest height, all drops were shielded from air currents during fall by a vertical asbestos-cement pipe (11 cm. internal diam.) attached to the Dexion tower. During an experiment pipettes were allowed to drip continuously on to a wooden or blotting-paper shutter which was removed only when a drop was required to hit the target film.

Target film. The target film normally consisted of a suspension of conidia of *Fusarium solani* in distilled water. Conidia were produced on cultures of potato glucose agar in 6 oz. medical flats. At the suggestion of Dr E. W. Buxton the cultures for some of the experiments were grown under continuous fluorescent lighting to suppress microconidia and obtain almost pure macroconidia. When the cultures were sporulating the conidia were washed off in 20 ml. water/2 medical flats, and the concentration of a 1/10 dilution determined with a haemocytometer. Two bottles normally gave a concentration of about 16×10^6 macroconidia/ml.

The target film was placed in a 6·6 cm.^{2} area marked with grease pencil on a microscope slide which had been cleaned by flaming after soaking in ethanol. Three film thicknesses were used, 0·1, 0·5 and 1·0 mm. The required volume of spore suspension was placed on the area from a pipette, care being taken to spread the film evenly because the thinnest films tended to contract under surface tension.

Because the conidia sank to the bottom of the film it was decided to have a constant number of spores/unit area of the target film. A concentration of 16×10^6 macroconidia/ml. was used for the film 0·1 mm. thick, and 1/5, and 1/10 dilutions for the 0·5 and 1·0 mm. films, respectively, all thus giving *c.* 160,000 macroconidia/cm.^{2} of target film. Each slide was normally used as target for one falling drop only (occasionally for two to five splashes by the smallest drops).

Sampling slides. The naphthol green B slides on which the splash droplets were caught were prepared according to the recommendations of Liddell & Wootten (1957). To purify the dye 10 g. of naphthol green B (British Drug Houses Ltd.) was dissolved in 50 ml. boiling water. On cooling it was salted out by adding 10 g. powdered anhydrous sodium acetate and allowing to stand for several days at 4° . The mixture was then filtered and the filter cake extracted with boiling absolute ethanol until the sodium acetate was removed.

Purified naphthol green B (1 g.) was dissolved in 20 ml. warm distilled water, filtered and added to 10 ml. of a 4% (w/v) solution of gelatine, and used to coat the slides on which splash droplets were caught. Microscope slides (3 × 1 in.) were washed in detergent and dipped in ethanol before polishing with a cloth. Two drops of naphthol green B gelatine solution were placed on the slide and spread evenly with the edge of another slide.

Slides for use near the target film had arcs 1 cm. apart drawn on the back. The arrangement of slides for sampling a splash is shown in Fig. 1. Slides were placed along one radius only, with the point of impact as centre, and arranged at intervals up to 100 cm. from the point of impact. Each experiment was normally based on five to ten separate splashes to avoid asymmetry in a single splash and to obtain enough droplets to give a reliable count at the greatest distances. After exposure the slides were scanned under a microscope provided with a mechanical stage, the area actually scanned being assumed to be a representative sample of its annulus (Fig. 1). The total number of droplets was estimated by multiplying by the appropriate factor. The path followed in scanning varied according to the proximity of the zone to the target area. The path is shown by the dotted line on Fig. 1. Each stain in the area scanned was measured and the number of macroconidia recorded (a value of over 60 macroconidia was classified as 'numerous').

Calibration of the naphthol green slide method. The method of calibration was based on that of Courshee & Byass (1953) in which a spinning disk apparatus was used to produce a spray of uniform droplets whose sizes were measured directly by catching a sample in oil. Other drops were caught on naphthol green slides and the size of the stain measured. A Perspex disk of diameter 4½ in. was fitted on a 1/20th horse-power high-speed motor and water fed on to the upper surface of the disk at a rate of 8 ml./min. The motor was mounted inside a box which also acted as a shield, and a narrow band of spray droplets emerged through a slot cut in the side of the box. We are indebted to Mr J. B. Byass of the National Institute of Agricultural Engineering who suggested the method and who lent a suitable motor for the spinning disk.

For the direct measurement of droplet sizes the droplets were caught on a layer of a 24% 'Vaseline' + 76% liquid paraffin (w/v) mixture and covered

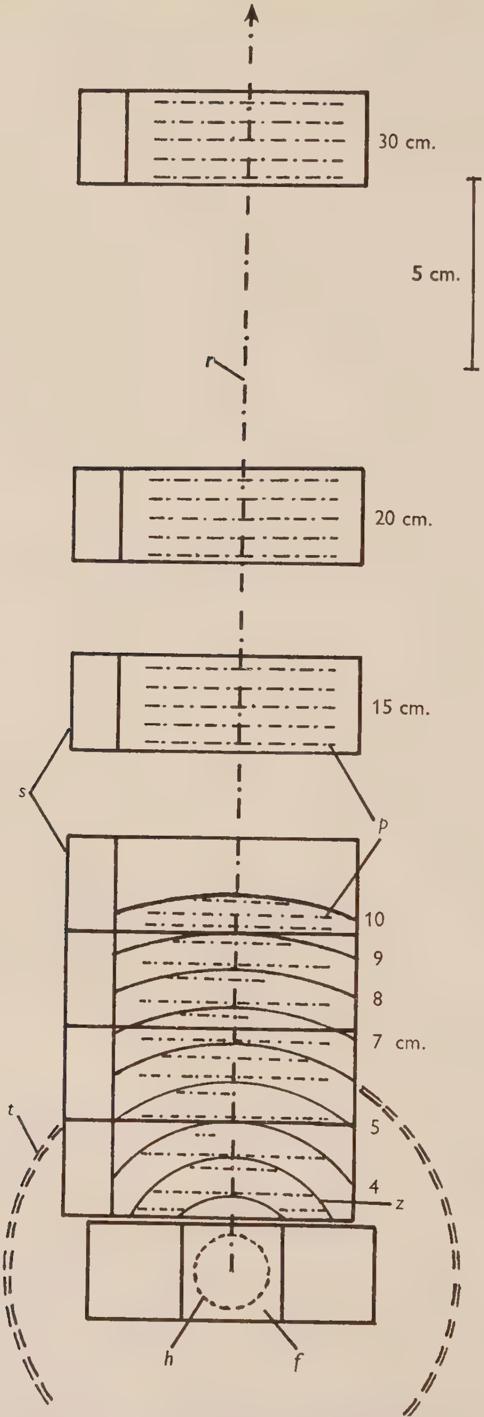
30 cm.
5 cm.
r
20 cm.
15 cm.
s
p
10
9
8
7 cm.
t
5
4
z
h
f

Fig. 1. Arrangement of target film, sampling slides and shields. *f* = target film; *h* = hole in Perspex shield; *p* = scanning path; *s* = sampling slides; *r* = radius from splash; *t* = asbestos pipe; *z* = zone limit.

as soon as possible (about 10 sec.) with pure liquid paraffin to prevent further evaporation.

As a first step to calibration evaporation curves were plotted for uncovered droplets of a range of sizes by focusing a microscope on the surface of the oil mixture in a measuring cell placed in the direct spray from the spinning disk. As soon as a droplet fell on the oil in the field of the microscope it was measured with a micrometer eyepiece and further measurements made at intervals of 15 sec. until the droplet had evaporated completely or had sunk into the oil. The sizes were then plotted and the % evaporation in the first 10 sec. for each size of droplet examined was obtained from the resulting graphs. These figures for % evaporation were then plotted (Fig. 2), from which the loss by evaporation in 10 sec. by a droplet of any size could be estimated.

In each calibration experiment the speed of the spinning disk was regulated by rheostats to give spray droplets of the required size. One cell containing a layer of the liquid paraffin + 'Vaseline' mixture, and two naphthol green slides were then exposed to the spray about 2 feet below the level of the disk. The droplets on the oil were covered with liquid paraffin as quickly as possible and the sizes of 10 droplets measured with a micrometer eyepiece. The observed size of the droplets was then corrected for evaporation as shown in Table 1.

Table 1. *Calculation of corrected droplet size*

Measured droplet diameter (μ)	Percentage evaporation E (from Fig. 2)	Value of $100/x$	Corrected droplet diameter (μ)	Stain diameter (μ)
129	3·9	104·1	134	396
181	3·1	103·2	186	530
328	1·6	101·6	333	1064
483	1·1	101·1	488	1503
707	0·8	100·8	713	2226
775	0·75	100·8	781	2435
836	0·7	100·7	842	2780
884	0·6	100·6	889	3266
887	0·6	100·6	892	3352
1018	0·5	100·5	1023	3566

Percentage evaporation after 10 sec. (E) was obtained from Fig. 2 and subtracted from 100 to give x.

Five splash droplet stains near the centre of each naphthol green slide were then measured in two directions at right angles. It was found that differences in the thickness of the naphthol green film could lead to differences of up to 10 % in the stain size, and care was therefore taken to use films of equal thickness as judged against a colour standard.

Measurements were made on droplets in the size range 100–1000 μ, the limits being set by the ability of the available spinning disk apparatus to produce spray of uniform-sized droplets. The disk would in fact produce droplets much smaller than 100 μ in diameter, but the turbulence created by the disk at high speeds mixed the droplets (normally separated by the effect of centrifugal force) and gave a spray of droplets of mixed sizes.

The difficulty at the upper limit was caused by fluctuations in the mains voltage. The motor was running at very low speeds when producing droplets of more than 1000 μ diameter, and was consequently very sensitive to alterations of voltage. Because no means of 'smoothing' the mains current was available to us the top end of the size range was not explored fully.

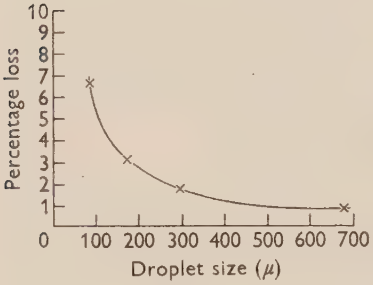

Fig. 2

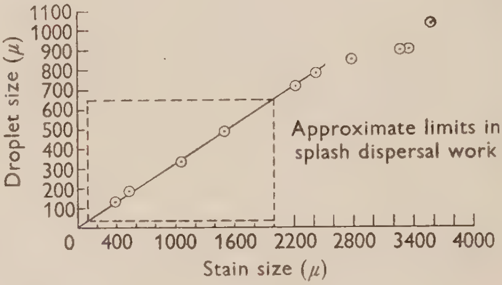

Fig. 3

Fig. 2. Percentage loss of diameter by evaporation in 10 sec. of droplets from spinning disk.

Fig. 3. Calibration of naphthol green slides with droplets from spinning disk. The relationship between stain size and droplet size.

When the corrected droplet sizes were plotted against stain sizes (Fig. 3), over most of the range the points lay on a straight line which passed through zero when extrapolated back. The mean stain size: droplet size ratio for the first six points on Fig. 3 is 3·094:1; the line drawn through these points is based on this ratio. The factor 0·32 (= 1/3·094) has been used to convert stain size to droplet size. For droplets of diameter >800 μ the relative size of the stain decreases, presumably because of evaporation and surface tension.

Performing the experiment. The pipette was adjusted for height and size of drop, and set to drip with the shutter in the closed position over the target. Beneath the shutter was a hole 2 cm. diam. (through which the drops were aimed) cut in a square of Perspex. The hole was surrounded by a pad of blotting paper to absorb any drops which missed the hole. The green slides were placed at predetermined distances along a radius, and finally the target film was placed under the hole. The target area was screened from draughts with Polythene sheeting. When all was ready the shutter was opened and one drop allowed to fall through the hole on to the target. The shutter was then closed while a new target film was placed in position and reopened for another drop to pass. Droplets from five splashes were collected on one set of naphthol green slides.

An experiment normally consisted of ten splashes, but all data are calculated for a *single splash*, and as we are concerned with dissemination of pathogens emphasis is on droplet *numbers*, rather than volume of liquid splashed.

RESULTS

Results from preliminary experiments

Preliminary experiments showed that the number and sizes of droplets produced by a splash were controlled by: (1) size of drop; (2) velocity of fall of drop (height from which it fell); (3) thickness of surface film (Fig. 4). The main series of experiments described in later sections was designed to explore the effect of these factors, and was performed after preliminary experiments had led to the technique being standardized.

The surface tension of the incident raindrop would normally be higher than that of the surface film containing mucilage. Variation in surface tension of the liquids over the range likely to be encountered in rain splash under field conditions did not affect numbers of droplets (Tests 1 and 2 in Table 2) or the distances travelled.

Table 2. *Preliminary tests of relation between thickness of surface film and number of droplets produced*

Incident drops 5 mm. diam. falling 2·9 m.

Test number	Surface film Thickness (mm.)	Surface film Composition	Estimated total number of droplets
1	0·1	Tap water	1085 (100 %)
2	0·1	Spore suspension	1055 (97 %)
3	0·2	Spore suspension	868 (80 %)
4	1·0	Spore suspension	462 (43 %)
5	Nil	Dry glass slide	27 (2·5 %)

As shown in Table 2 the number of droplets increased as the target film decreased in depth, but splash on a dry surface, as already noted by Faulwetter (1917*b*), produced very few droplets. Our data give no indication of the boundary between these conflicting trends and the experimental limit is set by the difficulty of producing very thin films of liquid for test.

Comparison between naphthol green slides and culture dishes. In two experiments both naphthol green slides and Petri dishes containing potato glucose agar were exposed simultaneously, alternating along two radii, the positions of dishes and slides being interchanged at each replicate of three or six drops. Target films, 0·15 mm. thick, contained 5 million spores/ml. of *Fusarium solani* macroconidia in one experiment and of *Colletotrichum lindemuthianum* in the other. After exposure the Petri dishes were incubated, and colonies counted on an illuminated screen every few hours. The highest count, before individual colonies merged, was taken as the total for the plate.

As indicated in Fig. 5, at 10 cm. distance the number of colonies was fewer than the number of drops; the reasons are discussed below. The numbers of droplets carrying Colletotrichum spores were consistently higher than those carrying Fusarium spores. A similar effect is discussed in the next paragraph.

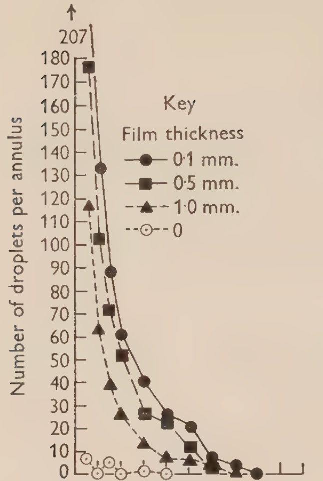

Fig. 4

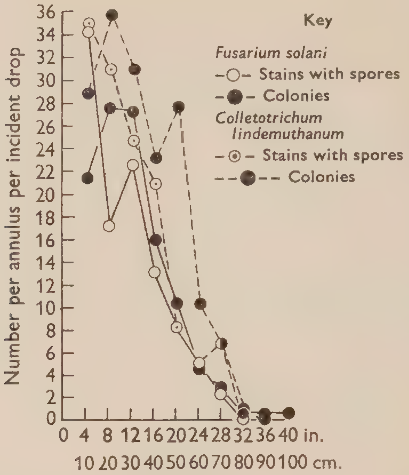

Fig. 5

Fig. 4. Effect of differences in thickness of target film on splashes from drops 5·0 mm. in diameter.

Fig. 5. Comparison of the numbers of droplet stains with spores on naphthol green slides with numbers of colonies on plates.

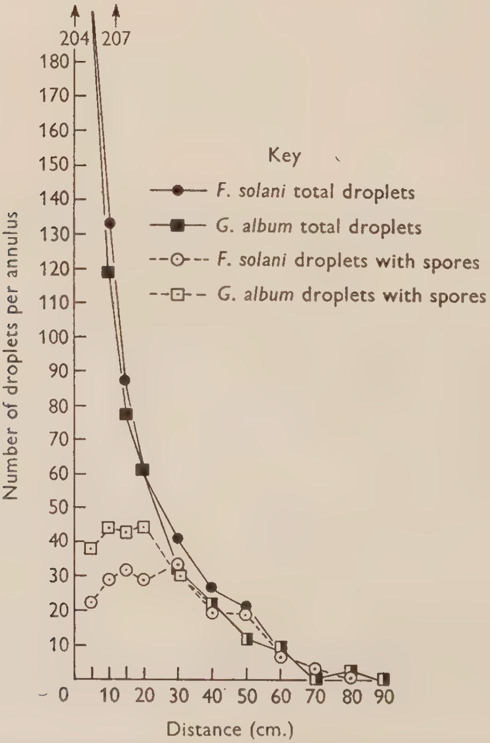

Fig. 6. Effect of variation in the spore type in the target film.

Transport of spores by droplets. Preliminary experiments with target films 0·1 mm. thick containing suspensions of macroconidia of *Fusarium solani* and conidia of *Gloeosporium album*, respectively, both at a concentration of 5×10^6/ml., are shown in Fig. 6. The incident drops in both experiments were 5 mm. diam. falling 2·9 m. Three drops were used in each test for distances up to 50 cm. and six drops for greater distances. Each test consisted of four replicates.

Spores of *Gloeosporium album* were carried by more splash droplets than those of *Fusarium solani*. The total numbers of droplets falling at distances up to 20 cm. were similar in both experiments, but spores of *F. solani* were carried by only 65 % of the number of droplets which carried *G. album*. The mean length of conidia in *G. album* is only about two-thirds of that in *F. solani*, and it appears that small spores such as those of *G. album* and *Colletotrichum lindemuthianum* are more easily picked up in splash droplets.

Experiments with bacteria. Preliminary tests with *Escherichia coli* were also made and numerous colonies obtained when Petri dishes of nutrient agar were exposed to splash. The results, however, were very irregular; colonies were often found in groups and it was difficult to determine whether some had come from one or from several droplets. Naphthol green slides were not exposed because of the difficulty of locating single bacteria during scanning.

Main series of experiments

The main series of experiments was designed to measure the effects of drop-size, film thickness and terminal velocity as obtained from different heights of fall on the formation and scatter of droplets.

Conditions fixed. Horizontal target film as source; number of macroconidia of *Fusarium solani*/unit area of target film, 16×10^4/cm.^{2}; still air, in laboratory screened from draughts; trapping surface horizontal, on level with target film, over distance 2–100 cm. from impact.

Conditions uncontrolled. Ambient temperature, humidity and pressure of the air; surface tension of tap water and spore suspension; electrostatic charge on drops, film, etc.

Conditions varied. Diameter of incident drop: 2, 3, 4 and 5 mm.; thickness of target film: 0·1, 0·5 and 1·0 mm.; height of fall of drop (controlling energy and velocity of drop at impact) 2·9 and 7·4 m.

Table 3 shows the estimated sizes and other properties of the drops actually used. The two heights of fall chosen were a compromise between the greater height required to allow the drop to attain its terminal velocity and the need to hit a small target after falling through air. Terminal velocities are estimated by plotting the data of Laws (1941), Gunn & Kinzen (1949), and Best (1950). Velocity on impact was estimated from Laws (1941, fig. 5). For simplicity diameters of drops will be given to the nearest millimetre.

Data from a typical experiment. The data obtained are illustrated by details of a typical experiment using the standard methods. The experiment chosen is that of an incident drop 5 mm. in diameter falling from a height of 7·4 m. on to a film 0·1 mm. thick—the drop with the greatest kinetic energy tested.

The estimated numbers of droplets produced in one splash are shown in Table 4. Although the number of conidia in each droplet was counted, the Table shows only the numbers of droplets with or without macroconidia.

Table 3. *Estimated diameters, terminal and impact velocities, and kinetic energies of incident drops used in main series of experiments*

Drop diameter Intended (mm.)	Drop diameter Actual (mm.)	Volume of drop (mm.^{3})	Terminal velocity (cm./sec.)	Estimated velocity on impact (cm./sec.)	Estimated velocity on impact % of T.V.*	Estimated kinetic energy (ergs.)
Height of fall 2·9 m.						
2	2·0	4·19	650	580	89	700
3	2·9	12·8	785	620	79	1,950
4	3·8	28·7	870	640	74	5,900
5	4·9	61·6	920	680	74	14,300
Height of fall 7·4 m.						
3	3·0	14·1	795	770	97	4,200
4	4·0	33·5	890	840	94	11,800
5	5·0	65·4	920	870	94	24,800

(* T.V. = terminal velocity.)

Total number of droplets (*Table* 5). The total number of droplets resulting from one splash increased consistently with increase in droplet size and increase in height of fall, but decreased with increasing film thickness; the number varied from about 180 to 5200. The number of droplets carrying spores varied even more, from 24 droplets/splash of a 2 mm. drop falling from a height of 2·9 m. on a thick film, up to over 2000 spore-carrying droplets from a 5 mm. drop falling at nearly its terminal velocity on to a thin film. The proportion of spore-bearing droplets varied from 14 to 47 %. For reasons discussed later the total number of droplets is probably underestimated.

Total volume of droplets. Table 6 gives an estimate of the total volume of droplets thrown up by one splash. The values are subject to large errors as droplet volumes were computed from mean diameters of a size category instead of from the individual stain sizes. The total is likely to be greatly affected by random errors in sampling a few larger droplets, some of which probably travelled beyond the limit of the range sampled. The total volume of droplets thrown up by a splash varied from one quarter to over twice the volume of the incident drop.

Droplet size spectrum. Droplet diameters were determined by measuring stains with the ordinary ocular micrometer linear scale. As usual in particle size studies the droplet diameters have been grouped for subsequent treatment into categories increasing by $\sqrt{2}$. The smallest droplets were in general most numerous and the voluminous data of the main series of experiments are most conveniently summarized by plotting the cumulative percentage of the number of droplets up to the limit of each size group on logarithmic probability paper (Fig. 7), fitting a freehand curve by eye and estimating the number median (50 % diameter). The 16 and 84 % points are also estimated (Table 7) to enable the geometric standard deviation of the population to be estimated by the method of Drinker & Hatch (see Green & Lane, 1957). Median number

Table 4. *Illustrative experiment. Estimated total number of droplets resulting from one splash showing interaction of droplet size and the distance of travel*

Incident drop diameter = 5 mm.; height of fall = 7·4 m.; target film thickness = 0·1 mm. Numbers in parentheses are droplets containing conidia.

Diam. of droplets (μ)	Annulus (cm.)														
	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–17·5	17·5–25	25–35	35–45	45–55	55–65	65–75	Totals
<15	41	58	20	46	9	9	—	—	—	—	—	—	—	—	—	183
16–21	49 (1)	60 (2)	58 (2)	120	69	45	24	3	—	—	—	—	—	—	—	428 (5)
22–27	29 (3)	28	42 (2)	76 (4)	81 (3)	81	72	9	—	20	—	—	—	—	—	438 (12)
28–33	22 (2)	26 (2)	24	34 (6)	60	51	69	33	—	—	—	—	—	—	—	319 (10)
34–39	15	24 (8)	16	26 (4)	27	48 (3)	15	33	26 (13)	20	—	—	—	—	—	250 (28)
40–45	7 (3)	18	16 (4)	20 (6)	3	18	39 (6)	24 (3)	130	20	—	—	—	—	—	295 (22)
46–57	11 (5)	8 (4)	16 (4)	32 (6)	33 (3)	18 (3)	24 (9)	33 (3)	143	40	—	—	—	—	—	358 (37)
58–82	14 (9)	16 (8)	18 (8)	36 (20)	27 (21)	30 (12)	45 (21)	27 (9)	169 (13)	200 (80)	—	—	—	—	—	582 (201)
83–116	10 (6)	12 (10)	14 (8)	16 (12)	15 (12)	9 (6)	18 (12)	42 (27)	130 (117)	140 (40)	76 (38)	50	—	—	—	532 (288)
117–164	11 (8)	10 (6)	12 (6)	6 (2)	9 (3)	6 (3)	24 (12)	15 (12)	91 (78)	100 (80)	76	50	—	—	—	410 (210)
165–231	6 (5)	8 (6)	6 (4)	8 (6)	9 (6)	3	21 (15)	24 (6)	52 (26)	80 (80)	38 (38)	100 (100)	—	150 (150)	—	505 (442)
232–328	10 (8)	4 (4)	4 (2)	6 (6)	6	12 (12)	9 (3)	12 (12)	26 (26)	20 (20)	—	—	63 (63)	75 (75)	88 (88)	335 (319)
329–464	10 (8)	3 (3)	2 (2)	8 (6)	12 (12)	3 (3)	—	6 (3)	39 (39)	20 (20)	76 (38)	100 (100)	—	—	88 (88)	367 (322)
465–655	1 (1)	—	10 (10)	2 (2)	—	3 (3)	3 (3)	3 (3)	26 (26)	80 (60)	—	—	—	75 (75)	—	203 (183)
>655	4 (4)	8 (8)	2 (2)	4 (4)	3 (3)	—	—	—	13 (13)	20 (20)	—	—	—	—	—	54 (54)
Totals	233 (58)	290 (66)	260 (54)	440 (84)	363 (63)	336 (45)	363 (81)	264 (78)	845 (351)	760 (400)	266 (114)	300 (200)	63 (63)	300 (300)	176 (176)	5259 (2133)

Table 5. *Estimated total number of droplets resulting from one splash*

Height of fall (m.)	Diam. of incident drop (mm.)	Target film thickness					
		0·1 mm.		0·5 mm.		1·0 mm.	
		Total droplets	Droplets with spores	Total droplets	Droplets with spores	Total droplets	Droplets with spores
2·9	2	309	82	172	26	175	24
2·9	3	828	348	563	109	487	82
2·9	4	1470	567	993	211	817	127
2·9	5	4110	1560	2470	829	1760	522
7·4	3	1180	559	1020	283	1030	470
7·4	4	4830	2010	3020	603	2040	391
7·4	5	5260	2133	3580	1690	2160	518

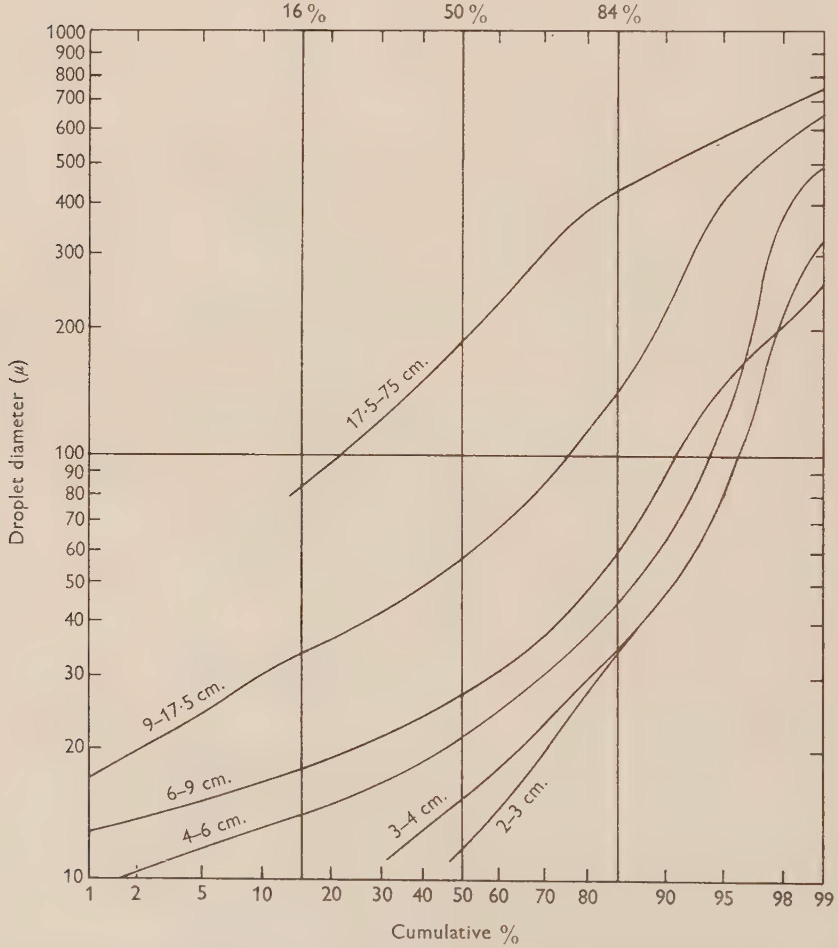

Fig. 7. Cumulative percentage of droplets plotted against increasing diameters shown at different distances for a splash from a drop 4 mm. in diameter falling 7·4 m. into a target film 0·1 mm. thick.

Table 6. *Estimated total volumes of droplets resulting from one splash*

	Incident drop		Target film thickness		
Height of fall (m.)	Diameter (mm.)	Estimated vol. (mm.³)	0·1 mm.	0·5 mm.	1·0 mm.
			Total vol. droplets from one splash (mm.³)		
2·9	2	4·2	1·6	2·4	2·8
2·9	3	12·8	4·9	3·9	9·7
2·9	4	28·7	12	12	8·6
2·9	5	61·6	58	56	37
7·4	3	14·1	3·8	13	29
7·4	4	33·5	50	27	41
7·4	5	65·4	53	140	60

Table 7. *Estimated median droplet diameter* (50%) *and geometric standard deviation* (μ)

		Target film thickness (mm.)								
		0·1			0·5			1·0		
Diam. of incident drop (mm.)		16%	50%	84%	16%	50%	84%	16%	50%	84%
		Diameters (μ)								
Height of fall 2·9 m.										
2	Total	24	115	330	23	55	230	22	55	200
	+spores	90	250	310	270	340	460	160	460	700
3	Total	22	66	240	16	42	150	17	64	360
	+spores	84	190	350	130	240	440	380	510	650
4	Total	19	62	235	21	50	180	18	55	140
	+ spores	70	160	460	105	290	600	185	220	600
5	Total	25	92	270	23	72	340	24	72	300
	+spores	80	240	480	110	310	600	120	260	700
Height of fall 7·4 m.										
3	Total	37	75	230	48	86	180	48	160	350
	+spores	85	190	400	88	180	450	160	220	550
4	Total	20	62	320	21	58	140	22	68	280
	+spores	115	210	400	82	160	400	230	480	750
5	Total	23	70	260	23	90	45	21	60	330
	+spores	60	140	300	130	300	800?	180	440	1000?

Total = values for total droplets. +spores = values for droplets carrying spores.

is stressed here, rather than the median volume usually preferred in aerosol studies, because the number of infective units is probably more significant in plant pathology.

Droplet diameters were estimated as varying from 5μ to about 2400μ. Under the conditions of the experiments few drops smaller than 30μ carried spores, the proportion decreasing with thick films. Median droplet size is slightly lower with large incident drops. The median size of droplets carrying spores appears to increase slightly with film thickness. The spore-transporting power of droplets can be expressed by extracting from the original data the mean diameters of those droplets which carry one (and only one) spore (Table 8).

Table 8. *Mean diameter (μ) of droplets carrying only one spore*

Target film with 160,000 spores/cm.^{2}

		Target film thickness (mm.)		
		0·1	0·5	1·0
Height of fall (m.)	Diam. of incident drop (mm.)	Mean diameter (μ)		
2·9	2	87	(318)	(163)
2·9	3	69	(56)	(60)
2·9	4	65	87	(92)
2·9	5	56	85	98
7·4	3	54	75	60
7·4	4	56	103	169
7·4	5	64	101	148
Calculated diameter of droplet of target film equivalent to 1 spore		70	118	150
Percentage of droplets containing only *one* spore which are less than 30μ diameter		9·5	2·6	1·6

Numbers in parentheses are means based on less than five droplets.

Horizontal travel of droplets in still air. The maximum horizontal distance to which droplets are splashed on the plane of the target in still air was about 100 cm. (Faulwetter recorded 125 cm.). Most droplets had a shorter trajectory and few reached 100 cm. (the limit used in the main series of experiments). The data are conveniently presented by the method used for droplet size, and the distances to which 16, 50 and 84 % of the droplets were thrown are entered in Table 9.

Table 9. *Horizontal distance of travel of droplets* (*all sizes*) *in still air*

		Target film thickness (mm.)								
		0·1			0·5			1·0		
Diameter of incident drop (mm.)		16 %	50 %	84 %	16 %	50 %	84 %	16 %	50 %	84 %
		Distance of travel (cm.)								
Height of fall 2·9 m.										
2	Total	3·5	8	20	3·5	8	20	3·5	8	20
	+ spores	4	8	20	?	?	?	4	15	?
3	Total	4	9	20	4	9	20	4	9	20
	+ spores	6	14	30	4	14	30	7	18	30
4	Total	4	9	20	4	9	20	4	9	20
	+ spores	5	13	30	4	11	30	4	9	20
5	Total	5	13	36	5	13	36	5	13	36
	+ spores	9	18	50	9	28	50	5	13	30
Height of fall 7·4 m.										
3	Total	6	13	33	5	10	31	8	21	32
	+ spores	7	14	30	5	14	23	7	21	34
4	Total	5	12	36	5	11	27	6	18	32
	+ spores	9	30	48	7	20	35	6	18	32
5	Total	5	10	35	5	10	23	5	9	23
	+ spores	7	21	55	6	14	28	5	13	26

The median horizontal distance travelled by droplets of all size categories varied from 8 to 18 cm. in the different experiments, increasing very little with the diameter of the incident drop, its height of fall, and the film thickness. Probably air resistance limits the flight of the small droplets.

Spore-carrying droplets were also insensitive to varying conditions. Their mean distance of travel tended to be greater than the mean for all droplets because very small drops tend to be spore-free and to travel only short distances. The greatest distances were travelled by medium size droplets (most of which carried spores under the conditions tested) especially in drops with the highest kinetic energy falling on to thin films.

Deposition/unit area on plane of target. The foregoing analysis of splash is relevant to the activity of raindrops in spore dispersal. Table 4 can be recalculated to give the average numbers of droplets deposited/cm.^{2} in the horizontal plane of the target, giving information relevant to the possibility of a healthy plant becoming contaminated from a nearby source in still air. Data for the 0·1 mm. films hit by various drops are given in Table 10. In still air the probability of contamination from a single splash decreases to a low value at the distance of 1 m.

Interaction of droplet size and horizontal travel. Droplets of different sizes had different maximum limits of horizontal travel, but apparently no minimum. The full data obtained on the relations between droplet size and distance of travel are illustrated in Fig. 7 by results of a typical experiment (drops 4 mm. diam. falling 7·4 m. on to a film 0·1 mm. thick.) Separate curves for five or six distances are plotted showing cumulative percentage of total droplets against increasing diameters. In this experiment, for example, 95 % of droplets falling 2–4 cm. from point of impact were less than 100 μ in diameter. At greater distances larger droplets predominate and only 20 % of the droplets falling beyond 17·5 cm. were less than 100 μ in diameter. Figure 7 is typical of the main series of experiments and the data reveal no clear trend with the various conditions tested. However, the absolute numbers of droplets (and of spore-carrying droplets) at each distance vary with the conditions; these data are summarized in Fig. 9.

Spore suspension in incident drop instead of in target film. Because of its bearing on the dispersal of spores in water dripping off foliage and splashing, the standard arrangement was reversed, using 5 mm. diam. drops of a suspension of 16×10^6 macroconidia of *Fusarium solani*/ml. falling 2·9 m. on to a target film of pure water. The droplets were caught over a representative range of 5–10 cm. from the target. In Table 11 their numbers are compared with those from the standard arrangement of water splashing on to a spore suspension in the film and show that splash droplets are composed of liquid from the incident drop and from the surface film.

Vertical travel

The vertical range of splash droplets was studied under one set of conditions only, using an incident drop 5 mm. diam. falling from 7·4 m. on to a film 0·1 mm. thick. Naphthol green slides were arranged vertically one above the

Table 10. *Number of droplets deposited/cm.^{2} in one splash*

Target film 0·1 mm. thick.

Drop diam. (mm.)	Distance from target (cm.)														
	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–17·5	17·5–25	25–35	35–45	45–55	55–65	65–75
Height of fall 2·9 m.															
2	2·46	1·91	0·945	0·670	0·541	0·530	0·286	0·151	0·122	0·028	—	—	—	—	—
3	3·75	3·37	2·790	1·59	0·958	1·14	1·03	0·490	0·280	0·080	0·040	—	—	—	—
4	7·45	5·45	5·14	3·74	2·06	1·78	1·26	0·982	0·380	0·200	0·080	0·020	—	—	—
5	12·3	8·37	6·54	6·28	5·18	3·65	2·46	1·78	0·780	0·700	0·360	0·120	0·160	0·040	—
Height of fall 7·4 m.															
3	2·74	2·18	1·98	0·990	1·26	0·890	0·858	0·528	0·233	0·260	0·060	0·040	0·020	—	—
4	19·2	13·4	8·23	8·30	7·22	5·02	5·27	2·37	1·20	0·539	0·281	0·159	0·100	0·039	—
5	14·8	13·2	9·81	12·1	8·92	7·12	6·93	3·31	1·30	0·759	0·140	0·119	0·020	0·079	0·039

Table 11. *Numbers and diameters of droplets when spore suspension is in incident drop compared with normal arrangement (suspension in target film)*

Incident drop 5 mm. diam. falling 2·9 m.

Conditions			Target film thickness (mm.)							
			0·1				1·0			
			No.	16 %	50 %	84 %	No.	16 %	50 %	84 %
Incident drop	Target film	Droplets		Diam. (μ)				Diam. (μ)		
Tap water	Spores 16×10^6/ml.	Total	876	20	31	80	462	23	34	300
		+ spores	200	57	96	350	118	140	400	1000
Spores 16×10^6/ml.	Tap water	Total	886	19	28	60	520	22	33	70
		+ spores	261	26	52	200	192	28	48	400

Table 12. *Numbers of droplets travelling further than selected distances as determined by vertical and horizontal slides*

Incident drop 5 mm. diam. falling 7·4 m. on to film 0·1 mm. thick.

	Vertical slides 0–40 cm. above target		Horizontal slides 2–100 cm. on plane from target	
Distance from source (cm.)	Total no. of droplets	Droplets carrying spores (% total)	Total no. of droplets	Droplets carrying spores (% total)
2·5	6340	24	5260	42
5·5	3580	24	4480	44
9·5	2920	53	2940	57
15	2200	51	2710	59
20	1360	62	1860	67
30	1880	82	1100	78
40	646	100	839	88
50	960	72	539	100
60	453	70	476	100

other on a frame to form a continuous test surface 40 cm. high with its base crossing the radius at right angles at a different distance in each of nine experiments. The total number of droplets/splash intercepted at the various distances was estimated by multiplying the number of stains observed by $360^\circ/\theta^\circ$ where θ° is the angle subtended by the sampling slide. In Table 12 these estimates of numbers of droplets intercepted are compared with the estimated numbers of droplets deposited beyond this distance in the appropriate experiment on horizontal travel. Median droplet height is shown in Table 13. The total numbers of droplets intercepted varied with height and distance as shown in Fig. 10.

Droplet diameters. Both the height and distance reached by droplets increased directly with increase in their diameter up to about 164μ. Maximum height, as well as horizontal range, appeared to be attained by droplets of intermediate size ranging from 164 to 328μ diam. (or even to 655μ). The few large droplets of approximately 1000–2000μ diam., which were observed when large incident drops fell at about 50 % of their terminal velocity into thick films, were mostly deposited within 3 or 4 cm. (Table 14).

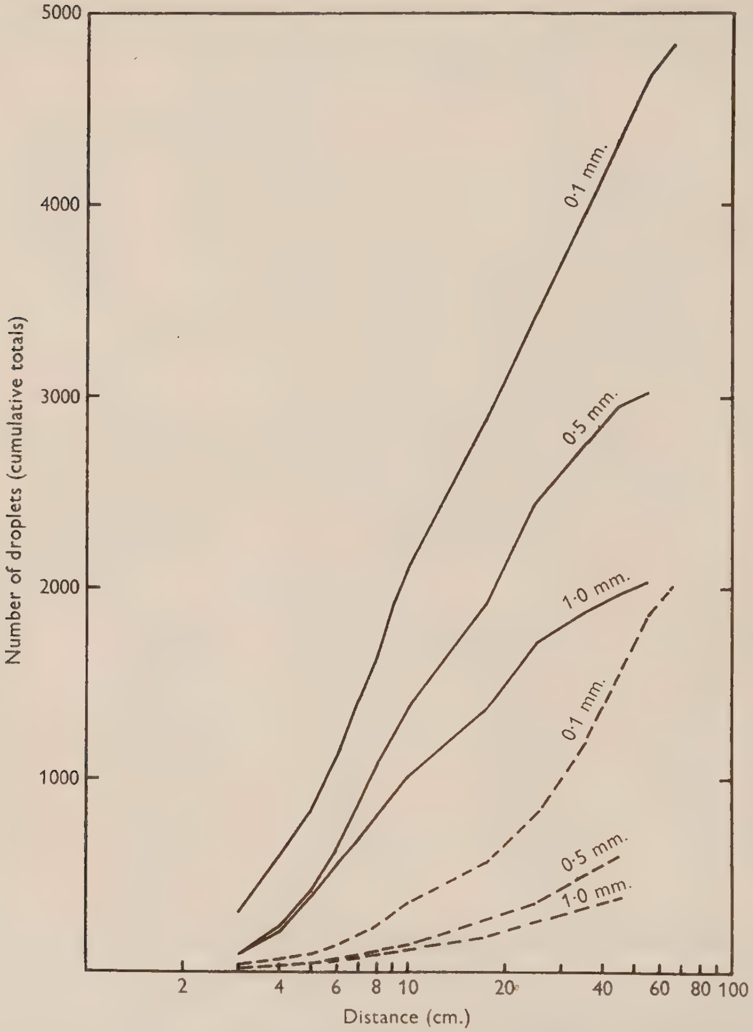

Fig. 8. Cumulative totals of all droplets (continuous lines) and droplets with spores (broken lines) from single splashes of drops 4 mm. in diameter falling 7·4 m. into target films of different thicknesses.

Splash on fungus fructification

The model studied in the main series of experiments is a relatively simple one and in the field pathogenic micro-organisms are exposed to rain splash at a complex variety of angles, roughnesses, sizes, etc. To test the relevance of our model to field conditions drops were allowed to splash on a twig of sycamore (*Acer pseudoplatanus*) bearing the pink cushion-like conidial fructifications of *Nectria cinnabarina*. The twig was supported at an angle of 45° at a height of 10 cm. above the sampling table. After wetting the twig in water, 5 drops, each 5 mm. in diam., were allowed to fall on the target from a height of 7·4 m.

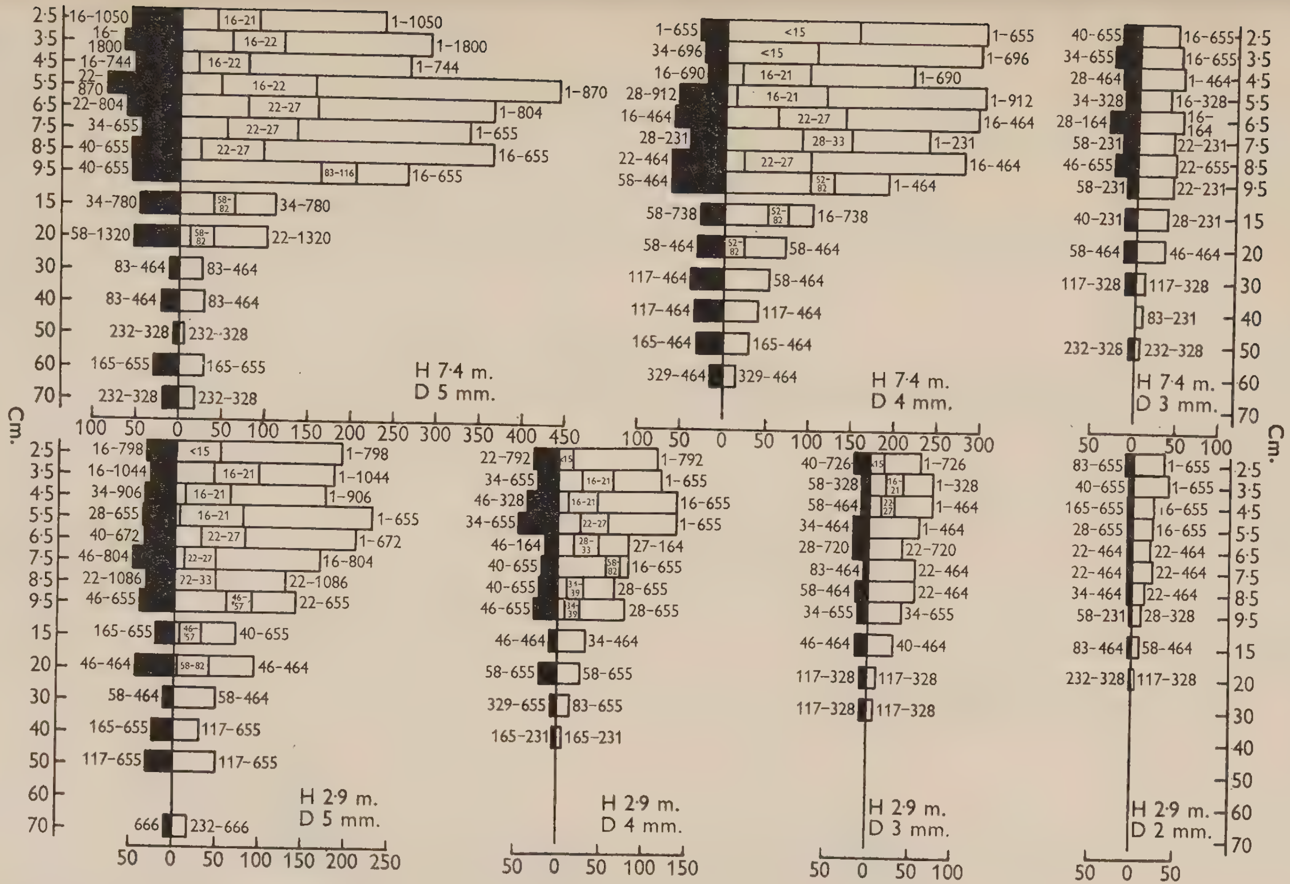

Fig. 9. Total number of droplets (white) and droplets with spores (black) in annuli 1 cm. wide from a single splash in a source film 0·1 mm. thick, with 16×10^4 macroconidia of *Fusarium solani*/cm.^{2} of surface. Distance shown to centre of annulus. Size-range of droplets (μ) in each annulus shown by figures outside each histogram. Size-range of modal class shown by figures inside white histograms.

Naphthol green slides were spaced in a rectangular pattern of rows and columns to sample splash in all directions up to a maximum distance of 50 cm. (Fig. 11). An estimated total of 2600 droplets was produced/incident drop. The size spectrum is compared with that from a thin film on a horizontal glass surface under similar conditions in Table 15. Splash from a wet twig at 45° produced both smaller and fewer droplets than splash from a horizontal slide (Fig. 12). However, *all* the droplets from the Nectria splash carried spores, mostly 10 or more. Figure 11 shows the numbers of droplets deposited/cm.^{2} in different positions for a single splash.

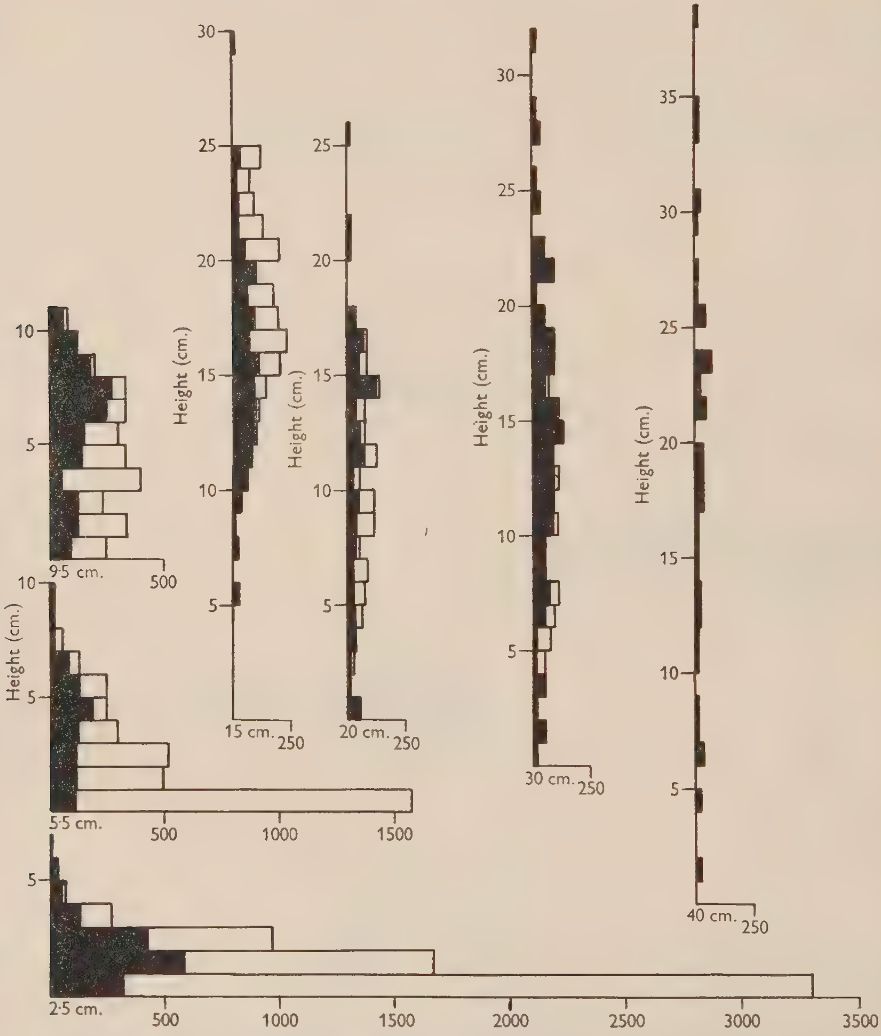

Fig. 10. Total number of droplets (black and white) and droplets with spores (black only) classified in heights at different distances from a single splash by a drop 5 mm. in diameter falling 7·4 m. into a source film 0·1 mm. thick with 160,000 macroconidia of *Fusarium solani*/cm.^{2} of surface.

Table 13. *Heights reached by droplets at selected distance from target (based on cumulative percentage)*

Distance (cm.)		16 % (cm.)	50 % (cm.)	84 % (cm.)
2·5	Total	<1	<1·0	2·3
	+spores	<1	1·6	3·0
5·5	,,	1·0	1·5	4·4
	,,	1·4	4·0	6·2
9·5	,,	1·7	4·8	7·8
	,,	2·3	6·4	9·0
15	,,	13·0	17·5	21·0
	,,	11·0	15·0	20·5
20	,,	5·0	11·0	15·5
	,,	5·0	12·0	16·5
30	,,	6·5	13·5	20·0
	,,	7·0	14·5	21·5
40	,,	8·5	19·5	26·0
	,,	8·5	19·5	26·0

Table 14. *Vertical and horizontal travel of droplets according to size*

Droplet diameters (μ)	Vertical height (cm.)		Horizontal range (cm.)	
	Mode	Maximum	Mode	Maximum
Values increasing with droplet diameter:				
10–40	≯2·5	2–17	2–8	0–30
40–164	5–7·5	17–25	10–25	0–45
164–655	25–35	30–40	55–75	0–75+
Values decreasing with droplet diameter:				
>655	10	25	17–25	0–75
±1000	—	—	2–3	0–17
±2000	—	—	2–3	0–7

Table 15. *Size spectrum of droplets produced by splash on twig (inclined at 45°) with conidial* Nectria cinnabarina *compared with splash on horizontal glass slide*

Incident drop 5 mm. diam., falling 7·4 m.

	16 %	50 %	84 %
Nectria twig	28μ	52μ	150μ
Glass slide	23μ	70μ	260μ

The course of splashes on another twig bearing conidial fructifications of *Nectria cinnabarina*, horizontally and at 45°, is shown by photographs (Pls. 1, 2, figs. 1–10) from a film made with the Kodak ultra high-speed camera by Mr E. D. Eyles of Kodak Limited for one of the authors (P.H.G.) in 1945.

DISCUSSION

The impact of a raindrop falling on a thin film results in many splash droplets being thrown into the air; the larger the incident drop the greater the number of droplets. The highest value observed, 5260 droplets/incident drop, is larger

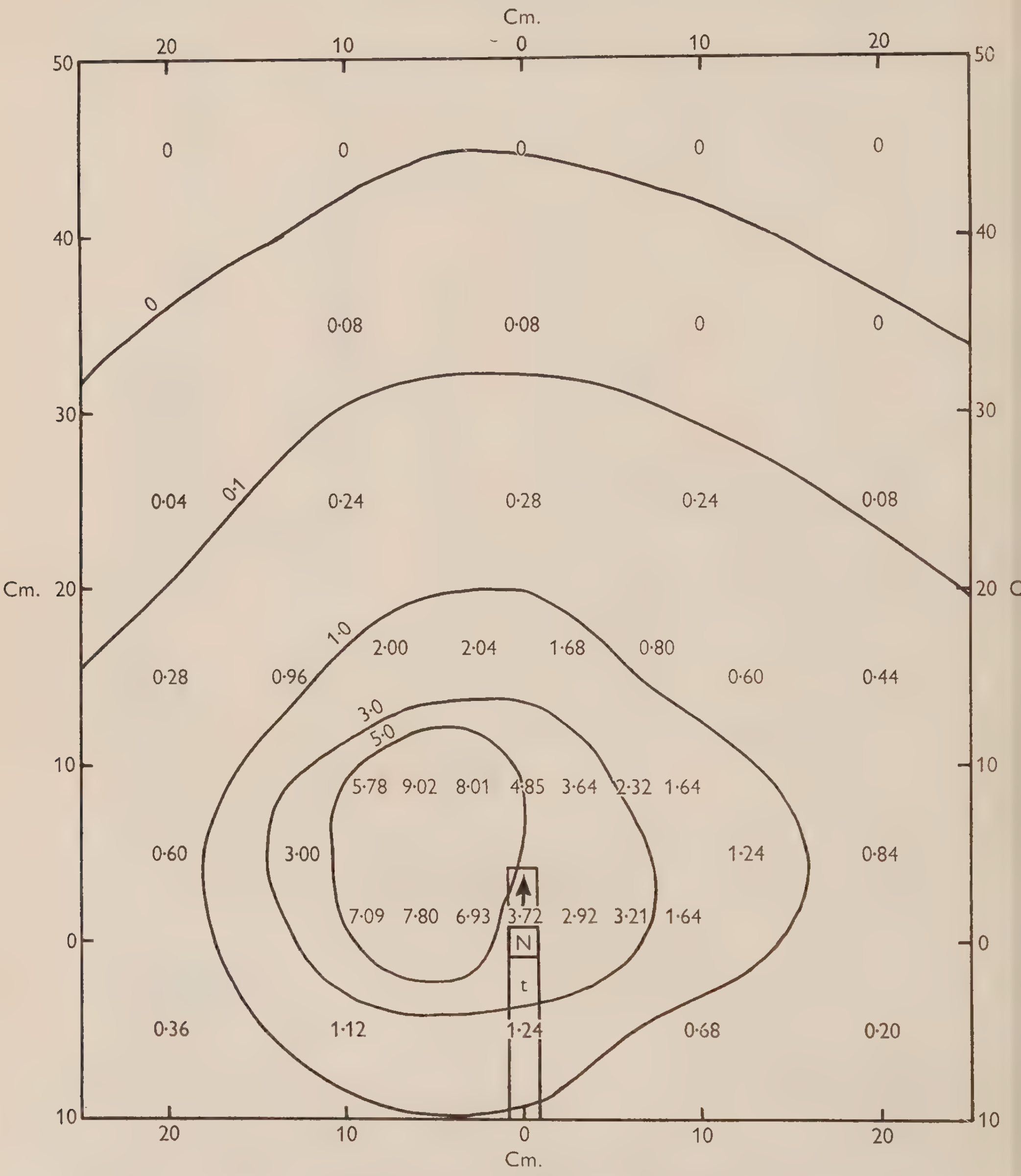

Fig. 11. Numbers of droplets/cm.² shown for one splash from a drop 5 mm. in diameter falling 7·4 m. on to a wetted twig of *Acer pseudoplatanus* bearing conidial fructifications of *Nectria cinnabarina*. t = twig; → shows the lower end of the twig; N = position of impact of drop.

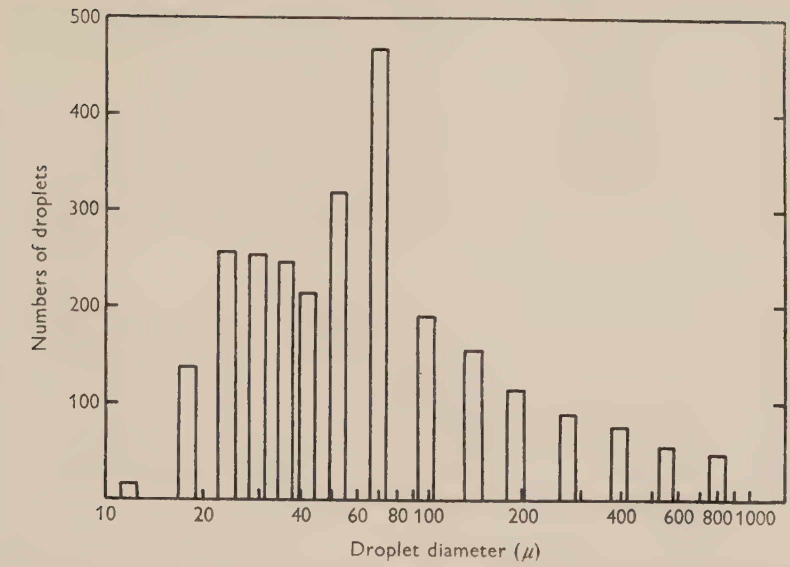

Fig. 12. Numbers of droplets classified in size-ranges from one splash from a drop 5 mm. in diameter falling 7·4 m. on to a wetted twig of *Acer pseudoplatanus* bearing conidial fructifications of *Nectria cinnabarina.*

than that reported in the pioneer work of Faulwetter (1917*b*), no doubt because we have used a microscope to scan very sensitive indicator slides. For the following reasons, however, even the high numbers obtained in these experiments are still probably underestimates. Any droplets smaller than those observed would probably evaporate during flight or, being too light to have a trajectory, would drift away on gentle air currents. A few droplets may have fallen beyond the 70 or 100 cm. radius sampled. Sometimes two or more droplets overlap on the film and are counted as one, particularly when larger droplets fall near the target. In still air of the laboratory others probably fall within 2 cm. or even on the target film although in moving air they might have functioned in spore dispersal. One factor which may have led to some over-estimation is the path taken in scanning the slides nearer the target; it might have been better, though more time-consuming, to sample random microscopic fields. Probably only the smallest drop-sizes are underestimated. The estimated numbers of larger drops carrying spores are probably unbiased but more subject to random errors when the small numbers falling at the greater distances have to be multiplied by a large factor to allow for the large area of the annulus.

Faulwetter (1917*b*) concluded that splash droplets consist almost solely of water from the surface film. As already reported (Gregory, 1952) it has become clear that the splash droplets are an intimate mixture of liquid from both the incident drop and the surface film. This can be demonstrated by placing a drop of green ink on a sheet of white paper and allowing a drop of red ink to fall on it from a height of 1–2 m. It is almost impossible to find a splash droplet of unmixed colour. Further, as Table 11 shows clearly, just as many splash droplets contain spores when the spore suspension is in the incident drop instead of in the surface film.

After a splash the target film shows a clear spore-free area several millimetres in diameter at the point of impact, sometimes surrounded by a ring of small cleared spots presumably at the base of larger rays of the splash crater. The rapid movement of liquid from the incident drop apparently sweeps the spores lying on the floor of the target film up the walls of the crater and into the droplets.

With the thinner films used in this work there is no evidence of bubble formation as described for splashes in deep layers of liquid by Worthington & Cole (1897), Blanchard & Woodcock (1957) and Mason (1957), but bubbles were observed in about one-third of the splashes caused by 5 mm. drops falling 7·4 m. into a deep film (1·0 mm. thick).

Splash dispersal in still air appears more local, producing a steeper gradient, than eddy diffusion of dry air-borne spores. The interior of a herb, bush or tree is a region where spore movement readily takes place in any direction by splash. A splash is of momentary duration, and dispersal appears unlikely to be further limited during rain by pick-up of droplets by raindrops. On the contrary, whereas a dry spore when once deposited is best considered 'out of play' as a first approximation, a splash-dispersed spore deposited on a wet surface is presumably susceptible to re-splash, so that continued rain may extend rather than limit the range of dispersal.

Dispersal by rain splash differs markedly from the more familiar 'take-off' mechanisms in dry air. Slime spores are held firmly to the matrix during dry periods and only set free in the kind of weather which is suitable for spore germination. The energy of raindrops is then available for their dispersal in splash droplets. The abundant smaller raindrops carry little energy and throw up few splash droplets. The larger, faster moving raindrops on the other hand are energetic producers of droplets. Spores carried in splash droplets during continued rain may be washed out of the air at once by other raindrops, or they may be washed off a surface on which they have been deposited. When carried by large droplets they may follow a definite trajectory, or when in small droplets they may behave after take-off like dry spores. The modification of the trajectories of splash droplets by wind was examined briefly by Faulwetter (1917*a*, *b*) but has apparently not been re-investigated since. Because they reach greater heights the droplets of medium diameter appear more likely to be carried off by wind.

While the largest raindrops falling at their terminal velocity appear most active in rain splash, experiments with fall heights between 2 and 3 m. show that much splash dispersal can also occur with large drops falling at only 50% of their terminal velocity. Such drops can occur both during overhead irrigation and under natural conditions in woodlands and within the frame of a single tree or bush. Further, leaves accumulate water and release large drops when wetted by mist, fog, and drizzle which all have drop sizes too small themselves to be significant splash producers. Drip from vegetation as well as impact of raindrops appears significant in spore dispersal by splash droplets (Faulwetter, 1917*b*; Gregory, 1952; Hirst, 1958). The mechanism is equally effective in dispersing spores either when water falls on to a film containing spores, or when a spore suspension from a leaf falls on to a spore-free surface film. Surface tension effects are likely to be significant in these circumstances.

Finally, splash dispersal can raise spores vertically. It is one of the mechanisms by which spores can be thrown into turbulent air and so remain in suspension for long periods. Alternatively, it is a method by which spores can climb upwards in a plant or a group of plants from a fructification lower down.

While macroconidia of *Fusarium solani* were readily removed from thin films in droplets of all diameters down to 50μ, few were found in droplets smaller than 30μ. It is not clear whether this lower limit is imposed by the size and shape of the spores or by the concentration of the spore suspension. The smaller spores of *Colletotrichum lindemuthianum* and *Gloeosporium album* are even more easily removed from thin films. Conidia of *Nectria cinnabarina* (*Tubercularia* state) and cells of *Escherichia coli* are very readily splashed. The ease with which spores of different shapes, sizes and surface are removed from various rigid and non-rigid plant surfaces requires study. So also does the splash from drops of 1 mm. diam. and less which are abundant in rain. The dispersal of micro-organisms by splash is not only feasible but is likely to be a highly effective process.

We wish to thank the Department of Scientific and Industrial Research for financial support for one of us (E.J.G.) and to thank: Mr J. B. Byass, National Institute of Agricultural Engineering, Silsoe; Mr E. D. Eyles of Kodak Limited; the staff of the Meteorological Department of Imperial College; and Messrs R. Adams and A. Horne of this Department for technical help.

REFERENCES

Best, A. C. (1950). The size distribution of raindrops. *Quart. J. R. met. Soc.* **76**, 16.

Blanchard, D. C. & Woodcock, A. H. (1957). Bubble formation in the sea and its meteorological significance. *Tellus*, **9**, 145.

Courshee, R. J. & Byass, J. B. (1953). Preparation and calibration of glass slides for sampling sprays. *Rep. Nat. Inst. agric. Engng Silsoe*, no. 32.

Edgerton, H. E. & Killian, J. R. (1939). *Flash: Seeing the Unseen by Ultra High-Speed Photography.* Boston: Hale.

Engel, O. G. (1955). Waterdrop collisions with solid surfaces. *J. Res. nat. Bur. Stand.* **54**, 281.

Faulwetter, R. C. (1917*a*). Dissemination of the angular leafspot of cotton. *J. agric. Res.* **8**, 457.

Faulwetter, R. C. (1917*b*). Wind-blown rain, a factor in disease dissemination. *J. agric. Res.* **10**, 639.

Green, H. L. & Lane, W. R. (1957). *Particulate Clouds: Dusts, Smokes and Mists.* London: Spon.

Gregory, P. H. (1952). Fungus spores. *Trans. Brit. mycol. Soc.* **35**, 1.

Gunn, R. & Kinzen, G. D. (1949). The terminal velocity of fall for water droplets in air. *J. Met.* **9**, 114, 394.

GUTHRIE, E. J. (1957). *A Study of the Rain-Splash Dispersal of Fungi.* Thesis for the Ph.D. degree of the University of London.

HIRST, J. M. (1958). Spore liberation and dispersal. (In the Press.)

LAWS, J. O. (1941). Measurements of the fall-velocities of water-drops and rain-drops. *Trans. Amer. geophys. Un. Rept. 22nd Ann. Gen. Mtg*, pp. 709–721.

LIDDELL, H. F. & WOOTTEN, N. W. (1957). The detection and measurement of water droplets. *Quart. J. R. met. Soc.* **83**, 263.

MASON, B. J. (1957). *The Physics of Clouds.* Oxford University Press.

NISIKADO, Y., INOUYE, T. & OKAMOTO, Y. (1955). Conditions of the spores of the scabbed wheat ear suspended in raindrops. *Ber. Ohara Inst.* **10**, 125.

STEPANOV, K. M. (1935). Dissemination of infective diseases of plants by air currents. *Bull. Pl. Prot., Leningr. Ser.* 2. *Phytopathology*, no. 8, pp. 1–68.

WORTHINGTON, A. M. & COLE, R. S. (1897). Impact with liquid surface studied by the aid of instantaneous photography. *Phil. Trans.* A, **189**, 137.

EXPLANATION OF PLATES

Photographs taken from an ultra high-speed film of a drop falling on a twig bearing conidial fructifications of *Nectria cinnabarina.*

PLATE 1

Twig at angle of 45°. Fig. 1. $t = -0{\cdot}006$ sec.; fig. 2, $t = +0{\cdot}003$ sec.; fig. 3, $t = +0{\cdot}012$ sec.; fig. 4, $t = +0{\cdot}021$ sec.; fig. 5, $t = +0{\cdot}033$ sec.; fig. 6, $t = +0{\cdot}054$ sec.

PLATE 2

Horizontal twig. Fig. 7, $t = 0$ sec.; fig. 8, $t = 0{\cdot}021$ sec.; fig. 9, $t = 0{\cdot}045$ sec.; fig. 10, $t = 0{\cdot}075$ sec.

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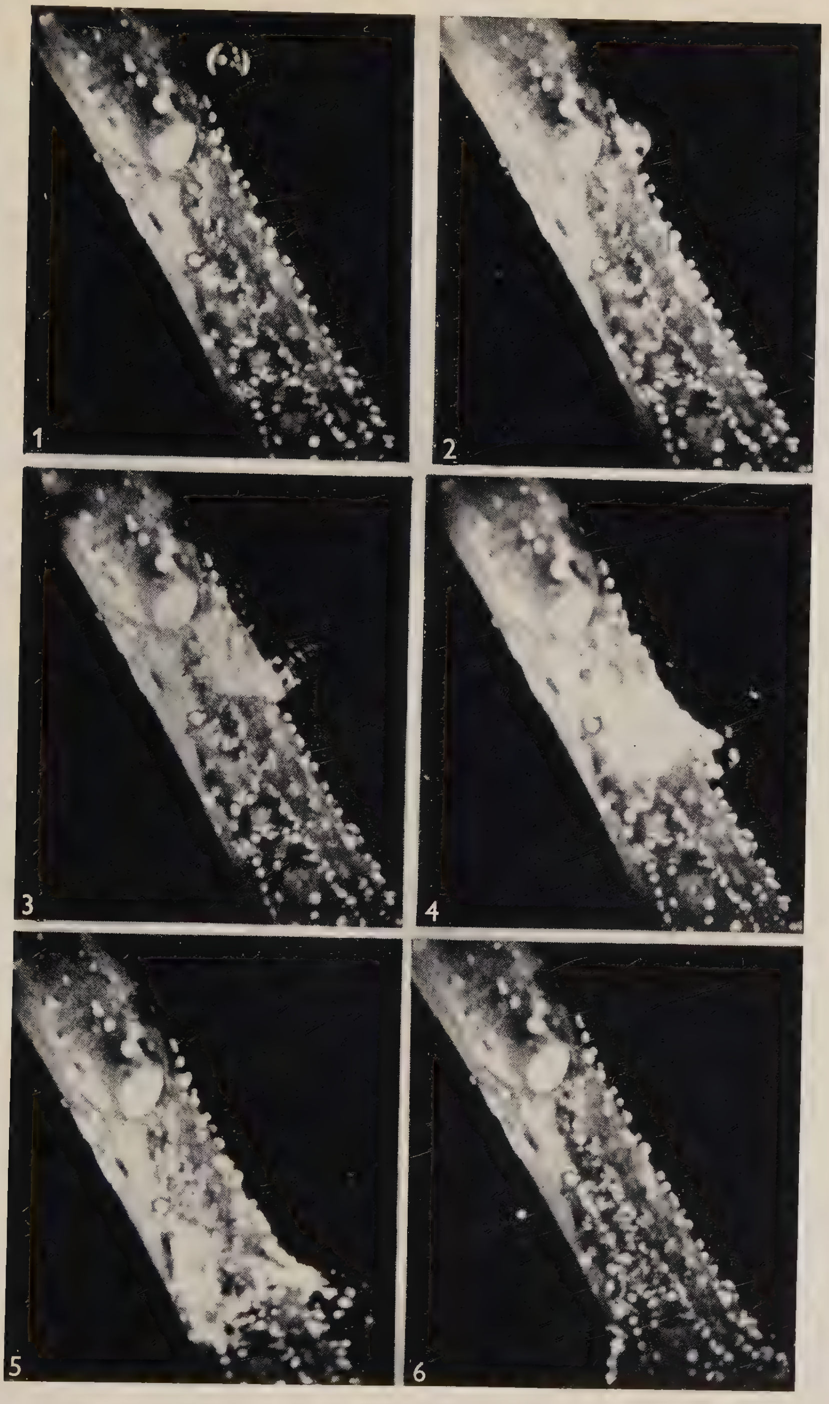
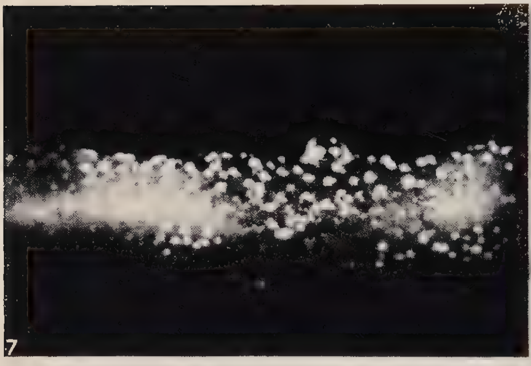


P. H. Gregory, E. J. Guthrie & M. E. Bunce—Splash dispersal of fungus spores. Plate 1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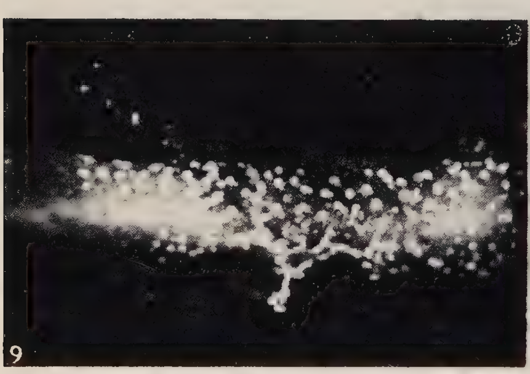
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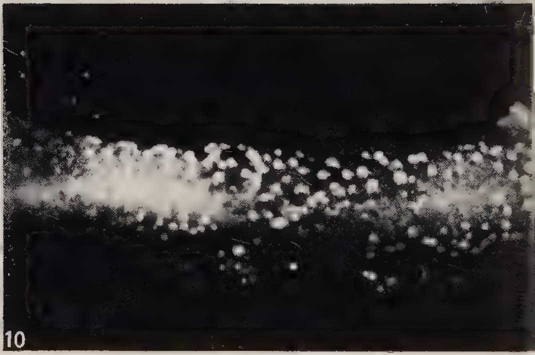
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P. H. GREGORY, E. J. GUTHRIE & M. E. BUNCE—SPLASH DISPERSAL OF FUNGUS SPORES. PLATE 2

Thornley, M. J. & Yudkin, J. (1959). *J. gen. Microbiol.* **20**, 355–364

The Origin of Bacterial Resistance to Proflavine

1. Training and Reversion in *Escherichia coli*

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SUMMARY: Several strains from *Escherichia coli* NCTC 8196 were isolated from discrete colonies. They were grown in liquid media in the presence of proflavine, and then in its absence. The distribution of resistance was measured by the number of organisms able to multiply at different concentrations of proflavine. Some strains were also grown in the presence of proflavine on plates, and the resistance of broth cultures from them was measured on gradient plates. The results cannot be explained on the assumption that the resistance was determined solely by specifically induced adaptation.

In this series of five papers we describe experiments designed to elucidate the mechanism by which cultures of *Escherichia coli* may acquire resistance to proflavine. We have chosen this substance because it was from work with it that Hinshelwood (e.g. 1946, 1949) has so strongly pressed the view that acquisition of drug resistance is through specifically induced adaptation rather than through mutation. One of us suggested that a third mechanism, clonal variation, may sometimes be involved (Yudkin, 1953). Many workers have thought that the reconciliation of these views lies in the existence of each of these mechanisms in different instances of acquired drug resistance. There is, however, a second possible way of reconciling the different mechanisms which have been put forward. This is that they may exist together in a single system of bacteria + drug, and that the mechanism which is found depends on the different experimental approaches made by different workers. We believe that we have been able to demonstrate this second possibility in the *Escherichia coli* + proflavine system. The work we describe in these papers brings evidence that, in this system, there is: (1) mutation and selection (Thornley & Yudkin, 1959); (2) a relatively small and reversible increase in resistance due to induction (adaptation) (Sinai & Yudkin, 1959*a*); (3) transformation of sensitive organisms to resistance by extracts containing deoxyribonucleic acid (DNA) from resistant organisms (Sinai & Yudkin, 1959*b*). Whilst we have not been able to demonstrate clonal variation, we believe that some of our results are best explained by assuming the existence also of this mechanism. We also have some evidence, though not yet conclusive, of a large and stable increase in resistance by induction.

In this paper, we describe experiments which were in the nature of a preliminary exploration of the problem. They were concerned with the changes in the resistance of cultures grown for varying periods in the presence of proflavine, and subsequently in its absence. They differ from the numerous experiments performed by Hinshelwood and his colleagues in two ways. These workers studied '*Bacterium lactis aerogenes*', and they measured changes in resistance by changes in the lag period in the presence of the drug. Most of our experiments were made with *Escherichia coli*, and in general we used the more usual criterion of resistance, the proportion of bacteria which can multiply in given concentrations of the drug.

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METHODS

Organism. We began with a culture of *Escherichia coli* NCTC 8196. It was maintained on nutrient agar slopes kept at 5° and subcultured at intervals of about two months. Several strains were isolated from discrete colonies, and were used in the experiments. Each of these has been given a different reference number.

Media. Two liquid media were used: a peptone broth of 1 % (w/v) peptone (Difco) in distilled water, and a nutrient broth containing 1 % (w/v) peptone (Difco), 0·5 % (w/v) sodium chloride and 0·3 % (w/v) 'Yeastrel' (Brewers' Foods Supply Co. Ltd.) in tap water, both at pH 7·0. Nutrient agar was made by adding 2 % (w/v) Japanese agar (British Drug Houses Ltd.) to the nutrient broth, and the pH value was adjusted to 7·4.

Incubation. All cultures were incubated at 37°.

Drugs. Proflavine was used as the neutral hemisulphate (British Drug Houses Ltd.); weighed portions were sterilized by heating to 100° for 1 hr., and dissolved in sterile medium. Chloramphenicol was dissolved directly in the medium, or used from concentrated stock solutions, without sterilization. No difficulty with contaminants was encountered.

Assessment of resistance. Resistance was measured in three different ways.

(1) *Dilution counts.* These were always made on overnight cultures grown without aeration at 37° in 1 % (w/v) peptone broth with or without proflavine. A series of 1/10 dilutions in tap water was made, then 0·5 ml. portions from appropriate dilutions were transferred to assay tubes containing 4·5 ml. peptone broth, or peptone broth containing different concentrations of proflavine. Each dilution was made into five replicate tubes, and those which gave visible turbidity after 48 hr. incubation were noted. This period was chosen because only occasionally was an additional positive tube obtained on more prolonged incubation, and this did not significantly affect the results. The reading of the number of positive tubes with three different dilutions of the inoculum (i.e. 'significant number') was converted to the 'most probable number'/ml. inoculum by the use of McCrady's tables (Buchanan & Fulmer, 1928). Where the assay tubes contained only broth, this number represented the total viable count of the culture under test. Where drug was included in the assay tubes, the number obtained was the most probable number of organisms/ml. able to multiply at that particular drug concentration. By using a range of about six different concentrations, figures were obtained showing the distribution of

drug-resistant organisms in the original population, where resistance is defined as the ability to multiply at a particular drug concentration. Since the action of proflavine is largely bacteriostatic, bacteria survived at concentrations much higher than those which caused growth inhibition.

(2) *Spread plate counts.* Like the dilution counts, these were always made on overnight 37° cultures in liquid medium. Plates of nutrient agar, alone or with added proflavine, were prepared and thoroughly dried. Serial 1/10 dilutions of the culture in tap water were made, and 0·1 ml. portions of the appropriate dilutions were transferred by a capillary blood pipette to each plate, and spread over the surface with a bent glass rod. Colony counts were made after various periods of incubation, but unless otherwise stated the counts recorded were those at 48 hr. Little or no increase in numbers took place after this time. The colonies were of fairly uniform size, so that there was no difficulty in counting them.

(3) *Gradient plates.* These were prepared according to the method of Szybalski (1952). Volumes of 10 ml. very hot nutrient agar were poured into a series of plates. This was allowed to set with one edge of the plates supported on a glass rod. In order to ensure an even slope and a correct direction of the gradient, a line was drawn ½ in. from one side of the plate, and on this line the plate was supported on the glass rod. The nutrient plates were now placed flat on the bench, and 10 ml. nutrient agar, containing the drug at the required concentration, poured on to the slope. To allow for diffusion of the drug and the drying of the agar, the plates were incubated overnight at 37° over $CaCl_2$. The plates so formed thus had a high concentration of drug at one end, which fell continuously across the plate to the other end, where it was zero.

From each broth culture to be tested was taken a standard loopful, and one streak made across the plate from the side with the highest concentration of drug. Up to 7 streaks, of different samples, were made on each plate. The plates were incubated overnight or for 48 hr. The usual appearance after incubation was solid growth part of the way across the plate, from the side with the lowest drug concentration. Beyond this, there was sometimes very thin dispersed growth, and near the side with the highest concentration there appeared a few large colonies. The resistance of a culture assessed in this way was determined from the length of the streak of solid growth, since this represented the resistance of at least a high proportion of the organisms. Thus, if on a plate containing 100 μg. proflavine/ml. solid growth occurred across one-third of the plate, the culture may be said to be resistant to *c.* 33 μg./ml.

The three methods were used for different types of experiment. In those concerned with training and reversion in liquid media, we desired to estimate the ability to multiply in liquid media. For these experiments therefore we used dilution counts. Similarly, for training on solid media, spread plate counts were used. Gradient plates were used for a rapid but less detailed assessment of the resistance of a large number of cultures.

The resistance measured by spread plate counts was apparently higher than that measured by dilution counts. This difference was some 20% with the sensitive strain assayed at low concentrations, but increased to some threefold for the strains of high resistance. It is possible that this may have been associated with a different solubility of proflavine in broth than in agar at high concentrations. The reproducibility of the spread-plate method is indicated in the next paper (Thornley & Yudkin 1959, figs. 1 & 2).

RESULTS

Training and reversion in liquid media

Each experiment was begun by plating the stock culture on to nutrient agar, incubating overnight at 37 °, picking off a discrete colony into 5 ml. broth and again incubating overnight. One loopful of this culture was then transferred into 5 ml. broth without or with a particular concentration of drug. Daily transfers were continued into broth with or without the drug for as long as was required for each experiment. At intervals, resistance was estimated by dilution counts on an 18 hr. culture.

Training. The resistance of sensitive strain 23 was tested immediately after isolation from a discrete colony, that is, after one growth period in broth, and again after 7 and 12 subcultures in broth (Fig. 1). In this and similar experiments, there was a tendency in later subcultures for an increase in numbers of the more resistant organisms, which would grow for example in 10 μg. proflavine/ml.

The effect of training was studied after one subculture in broth. An inoculum was made into broth with 5 μg. proflavine/ml., and dilution counts made after 1, 7 and 13 subcultures in the same concentration (Fig. 2). There was a considerable increase in numbers of resistant organisms, for example to 25 μg./ml., even after 1 subculture in drug, and a progressive increase with further subcultures.

Training in higher concentrations of proflavine was carried out after 1 subculture in broth, 2 subcultures in 5 μg./ml., and then repeated subcultures in 33 μg./ml. Measurements of resistance after 1, 7 and 15 subcultures in the higher concentration showed as before a great increase in numbers of highly resistant organisms, and progressive increase with further subcultures (Fig. 3). As well as strain 23, other strains were tested similarly and gave the same general picture but with differences in detail. In all instances, even one subculture in proflavine 5 μg./ml. or 33 μg./ml. gave rise to a high proportion of organisms able to grow at much higher concentrations than these.

Reversion after short training in 5 μg. proflavine/ml. Strain 22 was grown once in 5 μg. proflavine/ml. and then repeatedly in broth. After 5 subcultures there was reversion to a low resistance only a little higher than that of the original sensitive strain (Fig. 4). Very little further reversion occurred after 80 further subcultures.

Strain 20 was studied in a similar way. This gave a higher degree of resistance after 1 subculture in 5 μg. proflavine/ml. than did strain 22 after 2 subcultures. Subsequent growth in the absence of drug also gave much less reversion than was given by strain 22 (Fig. 5). There was slight reversion after

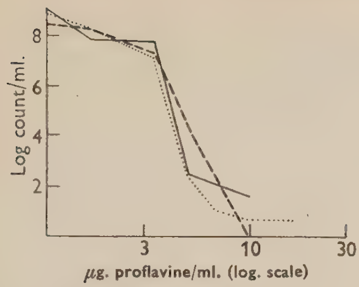

Fig. 1

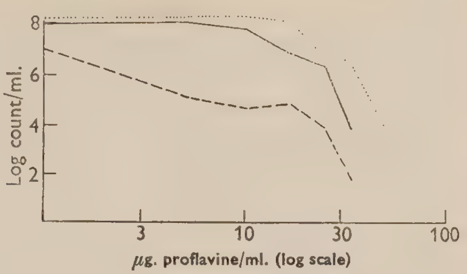

Fig. 2

Fig. 1. Distribution of resistance in sensitive culture of *Escherichia coli* 23, measured by dilution counts. These gave number of organisms/ml. capable of growing at given proflavine concentrations in broth. ---- = 1 subculture; ——— = 7 subcultures; ······ = 12 subcultures.

Fig. 2. Effect of growth in 5 μg. proflavine/ml. on resistance of *Escherichia coli* 23, measured by dilution counts. ---- = 1 subculture in proflavine; ——— = 7 subcultures in proflavine; ······ = 13 subcultures in proflavine.

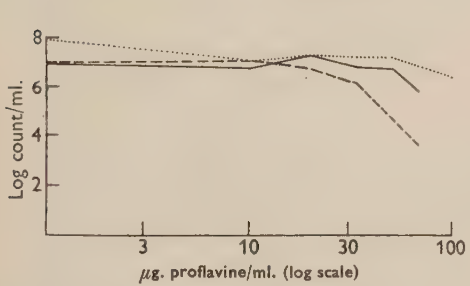

Fig. 3

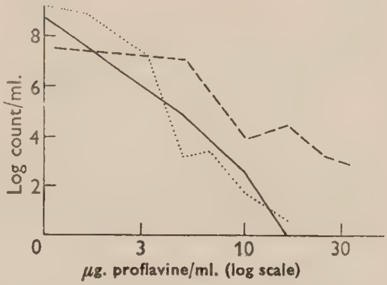

Fig. 4

Fig. 3. Effect of growth in 5 μg. proflavine/ml. for 2 subcultures, and then 33 μg./ml. for varying number of times, on resistance of *Escherichia coli* 23, measured by dilution counts. ---- = 1 subculture in 33 μg. proflavine/ml.; ——— = 7 subcultures in 33 μg. proflavine/ml.; ······ = 15 subcultures in proflavine 33 μg./ml.

Fig. 4. Reversion after training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown once in 5 μg. proflavine/ml., then in broth. ---- = 1 subculture in proflavine; ——— = 1 subculture in proflavine, then 5 times in broth; ······ = 1 subculture in proflavine, then 85 times in broth.

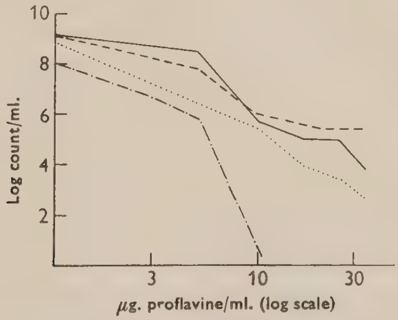

Fig. 5. Reversion after training. The resistance, measured by dilution counts, of *Escherichia coli* 20 grown once in 5 μg. proflavine/ml., then in broth. -·-·-· = grown in broth (original sensitive culture); ---- = 1 subculture in proflavine; ——— = 1 subculture in proflavine, then 4 times in broth; ····· = 1 subculture in proflavine, then 15 times in broth.

4 subcultures, and a little more after 15, but the resistance was still much higher than that of the original sensitive strain 20. Thus, complete reversion did not always occur on subculture in broth even when only 1 subculture in the presence of 5 µg. proflavine/ml. had been used for 'training'.

Reversion after prolonged training in 5 µg. proflavine/ml. Strain 22 was subcultured 12 times in 5 µg. proflavine/ml. and then repeatedly in its absence. There was a gradual decline in resistance (Fig. 6). Nevertheless, reversion was far from complete after 232 subcultures in the absence of drug. A very similar picture emerged after training for 84 subcultures in 5 µg. proflavine/ml. and subsequent subculturing in the absence of drug (Fig. 7).

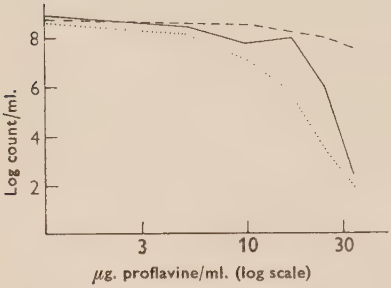

Fig. 6

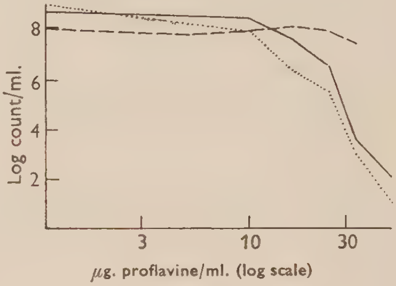

Fig. 7

Fig. 6. Reversion after prolonged training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown 12 times in 5 µg. proflavine/ml., then in broth. – – – – = 12 subcultures in proflavine; ——— = 12 subcultures in proflavine, then 42 times in broth; ······ = 12 subcultures in proflavine, then 232 times in broth.

Fig. 7. Reversion after prolonged training. Resistance, measured by dilution counts of *Escherichia coli* 22 grown 84 times in 5 µg. proflavine/ml., then in broth. – – – – = 84 subcultures in proflavine; ——— = 84 subcultures in proflavine, then 24 times in broth; ······ = 84 subcultures in proflavine, then 84 times in broth.

Reversion after training in 33 µg. proflavine/ml. Strain 22 was grown for 3 subcultures in 5 µg. proflavine/ml. and then once in 33 µg./ml. Subsequent subculture in the absence of drug gave a gradual decrease in the number of highly resistant organisms, but even after 175 subcultures without drug, the degree of reversion was small (Fig. 8). After more prolonged training, for 14, 29 or 84 subcultures in 33 µg. proflavine/ml., the pattern of reversion was similar to that seen after only one subculture in this concentration (e.g. Fig. 9).

Training on solid medium

In these experiments, broth cultures of sensitive strain 36 were spread on to plates containing varying concentrations of proflavine, over a range which prevented the growth of most of the organisms. From the colonies formed after incubation, inocula were transferred into broth, the tubes incubated overnight, and a loopful of the culture streaked on gradient plates.

Experiment 1. The sensitive strain was spread on 4 plates containing proflavine 3·3, 4, 5 and 6·7 µg./ml., respectively. The number of colonies which grew on each was noted at 24 and 48 hr. There was only a slight increase in numbers between these times (Table 1A). Both at 24 hr. and at 48 hr., 7–10 colonies were picked off each plate, grown separately overnight in broth, then streaked on gradient plates containing proflavine 10 μg./ml. or 67 μg./ml.

Most of the colonies grew either one-third and one-half across the plate with 10 μg./ml. (low resistance), or right across the plate with 67 μg./ml. (high resistance). One colony showed an intermediate resistance, growing about one-half across the plate with 67 μg./ml. Finally, some colonies gave growth across the plate with low proflavine concentration but only scattered colonies on the plate with high concentration. These are called colonies of unclassified resistance, and will be discussed below.

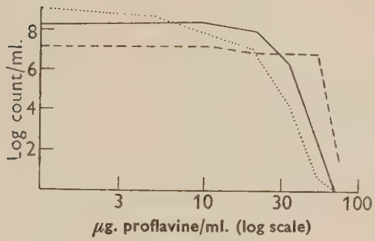

Fig. 8

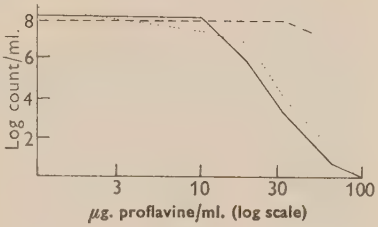

Fig. 9

Fig. 8. Reversion after training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown 3 times in 5 μg. proflavine/ml., then once in 33 μg./ml., then in broth. ––––= 1 subculture in 33 μg. proflavine/ml.; ——— = 1 subculture in 33 μg. proflavine/ml., then 22 times in broth; ······ = 1 subculture in 33 μg. proflavine/ml., then 175 times in broth.

Fig. 9. Reversion after prolonged training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown once in 5 μg. proflavine/ml., then 84 times in 33 μg./ml. then in broth. –––– = 84 subcultures in 33 μg. proflavine/ml.; ——— = 84 subcultures in 33 μg. proflavine/ml., then 24 times in broth; ······ = 84 subcultures in 33 μg. proflavine/ml., then 84 times in broth.

After 24 hr. of incubation on the gradient plate, 19 colonies showed low resistance and 11 high resistance (Table 1B). After 48 hr. the proportion of colonies of low resistance fell, there being 6 of these, 1 of intermediate resistance and 30 of high resistance. Since very few new colonies had developed between 24 and 48 hr., the increased proportion of resistance amongst the colonies must have developed during the further period of incubation. This change in proportion is discussed below.

Experiment 2. A sensitive culture was spread on to plates containing 10 μg. proflavine/ml. The colonies formed represented 1 in 2×10^6 of the organisms of the inoculum and of these colonies 79 were picked off and grown overnight in broth. Streaks were then made on gradient plates containing 100 μg. proflavine/ml. After 24 hr. of incubation, 76 of the cultures gave streaks representing resistance to more than 100 μg. proflavine/ml., and the other 3 gave streaks representing resistance to about 80 μg./ml.

Table 1. *Effect of training of* Escherichia coli 36 *on solid medium containing proflavine*

A. Number of colonies appearing on spread plates containing varying concentration of proflavine, inoculated from broth culture.

Proflavine concentration on which colonies were grown (µg./ml.)	Colony count/ml. inoculum after incubation for	
	24 hr.	48 hr.
0	$7\cdot8\times10^{8}$	$7\cdot8\times10^{8}$
3·3	$8\cdot1\times10^{7}$	$8\cdot2\times10^{7}$
4	$4\cdot4\times10^{7}$	$4\cdot5\times10^{7}$
10	$3\cdot6\times10^{2}$	$4\cdot4\times10^{2}$

B. Between 7 and 10 colonies growing on each proflavine concentration at 24 and 48 hr. were tested on gradient plates with proflavine 10 and 67 µg./ml.

Proflavine concentration from which colonies obtained (µg./ml.)	Number of colonies of indicated resistance*							
	24 hr.				48 hr.			
	3–5 µg./ml.	33 µg./ml.	> 67 µg./ml.	Unclassified	3–5 µg./ml.	33 µg./ml.	> 67 µg./ml.	Unclassified
3·3	7	0	0	3	2	0	7	1
4	7	0	1	2	4	0	4	1
5	5	0	1	1	0	1	9	0
10	0	0	9	0	0	0	10	0
Total	19	0	11	6	6	1	30	2

* Expressed as approximate concentration of proflavine at end of streak on gradient plate.

Note. Colonies grown in absence of proflavine always give streaks indicating resistance of 3–5 µg./ml.

DISCUSSION

The experiments of training and reversion in liquid media were similar to those reported by Hinshelwood and his colleagues, except that we measured the resistance of the resulting populations more exactly than by Hinshelwood's method of lag periods. Hinshelwood reported two main conclusions: (i) the effect of training was to give a resistance of about that represented by the concentration of drug to which the culture was trained; (ii) the rate of reversion was proportional to the period of training, being very rapid after short periods of training, and slower but still ultimately (nearly) complete after prolonged periods of training.

We cannot confirm either of these conclusions. In our experiments, a considerable proportion of organisms reached a resistance much greater than that represented by the concentration of drug used for training. Further, even after only 1 subculture in drug, reversion was not always complete after 14 or 15 subcultures in absence of drug. After a few transfers in the presence of only 5 µg. proflavine/ml., 232 subcultures in the absence of drug did not give complete reversion. Similarly, after 1 subculture in the presence of 33 µg./ml., 175 subcultures in the absence of drug did not give complete reversion. There was almost no difference in the rate of reversion after 12 subcultures in the presence of drug and after 84 subcultures. According to Hinshelwood's model, one would expect a shift in enzyme balance, characteristic of either sensitive or of resistant (adapted) organisms, to take place with similar ease in either direction. We have seen, however, that only short periods in the presence of drug give relatively high resistance, whilst subsequent quite long periods in its absence do not give complete reversion to sensitivity.

One could more readily explain the results on the assumption that spontaneous mutation to drug resistance occurs, and the mutant organisms are then selected in the presence of drug. We can then also explain the variation which we have observed in the behaviour of different strains, or more strictly different one-colony isolates from the parent strain, *Escherichia coli* NCTC 8196. First, the mutants will appear at random times. Secondly, different strains may well give different rates of mutation. Thirdly, the sensitive and the mutant organisms may have different growth rates. We shall in subsequent papers show that spontaneous mutations do occur, and that other mechanisms may also play a part in the overall behaviour of cultures grown in the presence and absence of the drug.

In three experiments on training on solid media such as we have described, 170 strains were examined from colonies grown on different amounts of proflavine in agar. Of these, 26 had a resistance similar to that of the original strain, 132 a very much higher resistance, and 12 gave scattered colonies. These last are discussed below. According to Hinshelwood's view, we should have expected that most of the organisms would have become adapted to about the degree of resistance represented by the concentration of proflavine on which the colonies had been grown. Against this view there are the following facts: (i) about 15 % of the colonies showed no increase of resistance; (ii) no colonies gave streaks indicating resistance to the concentration of proflavine on which they had been grown; (iii) most of the colonies gave streaks indicating an increase of resistance of at least ten times. It might be supposed that these streaks were produced by a combination of a large number of organisms adapted in the Hinshelwood manner, and a small number of highly resistant organisms derived, for example, from mutants. If this were so, the proportion of highly resistant organisms must be more than 10 %, since we have found that a smaller proportion gives a streak with scattered colonies. Thus, even if adaptation occurs, its effect is masked by the presence of at least 10 % of highly resistant organisms.

The few streaks which showed scattered colonies almost certainly represented a mixture of sensitive and resistant organisms. An exactly similar type of streak is produced from an artificially prepared mixture of organisms from sensitive and resistant strains. Such a mixture would occur if a mutation took place on the drug plate during the growth of the original colony before it was picked off for testing. As these colonies were developing in the presence of proflavine, it would be expected that the mutant organisms would in due course outgrow the sensitive organisms of the colony. This would account for our finding that the proportion of colonies showing high resistance increased between 24 and 48 hr. of incubation on the drug plate.

It is apparent that experiments of this nature do not allow any certain conclusions to be drawn as to the origin of the resistant organisms which arise during training. We feel, however, that we can perhaps draw one negative conclusion, which is that the results cannot easily, if at all, be explained solely on the basis of the Hinshelwood model of specifically induced adaptation. Our subsequent investigations therefore were designed to examine under more critical experimental conditions the possible modes of origin of proflavine resistance in *Escherichia coli*.

We are grateful to the Medical Research Council for a personal grant to one of us (M.J.T.).

REFERENCES

Buchanan, R. E. & Fulmer, E. I. (1928). *Physiology and Biochemistry of Bacteria*. London: Baillière, Tindall and Cox.

Hinshelwood, C. N. (1946). *The Chemical Kinetics of the Bacterial Cell*. Oxford: Clarendon Press.

Hinshelwood, C. N. (1949). Adaptation of bacteria to resist drug action (with special reference to *Bact. lactis aerogenes*). *Symp. Soc. exp. Biol.* **3**, 243.

Sinai, J. & Yudkin, J. (1959*a*). The origin of bacterial resistance to proflavine. 4. Cycles of resistance in *Escherichia coli* and their bearing on variations in resistance in cultures. *J. gen. Microbiol.* **20**, 384.

Sinai, J. & Yudkin, J. (1959*b*). The origin of bacterial resistance to proflavine. 5. Transformation of proflavine resistance in *Escherichia coli*. *J. gen. Microbiol.* **20**, 400.

Szybalski, W. (1952). Gradient plate technique for study of bacterial resistance. *Science*, **116**, 46.

Thornley, M. J. & Yudkin, J. (1959). The origin of bacterial resistance to proflavine. 2. Spontaneous mutation to proflavine resistance in *Escherichia coli*. *J. gen. Microbiol.* **20**, 365.

Yudkin, J. (1953). Origin of acquired drug resistance in bacteria. *Nature, Lond.* **171**, 541.

(*Received* 21 *October* 1958)

THORNLEY, M. J. & YUDKIN, J. (1959). *J. gen. Microbiol.* **20**, 365–372

The Origin of Bacterial Resistance to Proflavine

2. Spontaneous Mutation to Proflavine Resistance in *Escherichia coli*

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SUMMARY: Experiments were performed to demonstrate the production in *Escherichia coli* of spontaneously occurring mutants resistant to proflavine. The fluctuation test of Luria & Delbrück (1943) suggested that such mutants arise. This was confirmed by a modification of the technique of Lederberg & Lederberg (1952). By this method of indirect selection by replica plating, two separate first-step mutants were isolated; from one of these was isolated a second-step mutant, and from this a third-step mutant. The resistance of the third-step mutant was about 100 times greater than that of the original sensitive strain. By the same technique, two first-step mutants to chloramphenicol resistance were isolated. None of the 6 mutants had come into contact with either drug until the time of testing. Cross-resistance between proflavine and chloramphenicol was shown by 5 of the 6 mutants; the sixth showed an increase in proflavine resistance though not in resistance to chloramphenicol.

We have used two methods to demonstrate that mutants to proflavine resistance may arise spontaneously in cultures of *Escherichia coli*. The first was the fluctuation method of Luria & Delbrück (1943). This test, however, has been the subject of justifiable criticism (Eriksen, 1949; Dean & Hinshelwood, 1952). In particular, it has been pointed out that minor differences in environment can account for apparent fluctuations in numbers of resistant organisms. Our own results showed rather small fluctuations which might possibly have been accounted for in this way. We have therefore also used the more conclusive indirect selection method of Lederberg & Lederberg (1952).

METHODS

Organism. In all the experiments described in this paper we used strain 36 derived by us from *Escherichia coli* NCTC 8196. It was originally grown by us from a single colony, and had been transferred through a large number of broth cultures before the experiments began.

Media. The media and cultural conditions were the same as those described in the previous paper (Thornley & Yudkin, 1959).

Indirect selection by replica plating. Our method was a slight modification of that of Lederberg & Lederberg (1952). A nutrient agar plate was inoculated with 10^6–10^7 organisms of a sensitive strain and incubated at 37°. This formed the master plate for the first set of replicates. The incubation period was of importance; in early experiments 24 hr. was used, but later a 5 hr.

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incubation period was found to give better results (see Discussion). A square of velveteen, sterilized by autoclaving, was mounted with the pile upwards on a rubber bung 7 cm. in diameter, by means of a rubber band. The velvet had a triangle as a marker stitched in the centre. The master plate was pressed on the velvet pad, so that some of the bacteria were transferred to corresponding positions on the velvet, the position of the marker being traced on the back of the plate with grease pencil. A series of plates with increasing concentrations of proflavine was pressed in turn on the velvet pad, and the position of the marker traced on each. These drug plates were incubated for 24 hr. and, in the meantime, the master plate was kept at 2°. Colonies growing on the drug plates could be traced back to the corresponding area of the master plate by the system of markers. Where colonies grew at the same position on two or more proflavine plates of different concentrations, the area on the master plate was presumed to contain a microcolony of resistant organisms. An inoculum was taken from this point with the tip of a straight 'Nichrome' wire inoculating needle, and transferred to a new master plate for the second set of replicas. This inoculum was spread over this master plate by a bent glass rod. In the earlier experiments, an intermediate subculture was made from the inoculum into broth, and the new master plate was inoculated from this after overnight incubation.

The second set of replicas was carried out in exactly the same way as the first, including (in later experiments) the 5 hr. incubation period for the master plate. By means of these 2 cycles of replication, the proportion of resistant organisms was considerably increased, and therefore the master plate for the third set of replicas was usually inoculated in a different way. This was by streaking with the wire needle in a criss-cross pattern, giving fine lines of growth after 5 hr. of incubation. This modification, suggested by Dr Jehudith Sinai, enabled resistant microcolonies to be located more accurately. Under a low-power binocular microscope, a very small inoculum was picked from the appropriate position on the streak on the third master plate with a sharpened steel needle, and placed on the next master plate where it was spread over the surface by a bent glass rod. It was essential that the amount transferred should be sufficiently small to give not more than about 100 widely spaced colonies. This master plate (4th set) was incubated for 5 hr., the replicas on drug agar were made, and the master plate was re-incubated overnight. The final step in the process was to pick off a resistant colony from this master plate. As the colonies were well separated, the incubation could be prolonged to 24 hr. before picking off, without any loss of resolution, and with the obvious advantage that colonies were clearly visible, whereas 5 hr. microcolonies were very difficult to locate when widely scattered. It was still desirable, however, to use only 5 hr. of incubation before making the replicas (see Discussion).

Cultures of the resistant strain finally obtained were plated out and picked from discrete colonies twice, in order to make reasonably sure that they were in fact pure strains. From the final discrete colony, inoculation was made on to several slopes, and these formed the culture of each strain.

Assessment of resistance. The same three methods were used as in the preceding paper. These were dilution counts, counting on spread agar plates, and measuring the length of the streak on gradient plates. Dilution counts could not be used for the more resistant strains, since concentrations of proflavine greater than 100 μg./ml. reacted with the peptone solution, which became turbid. This reaction did not occur in the solid medium at concentrations of proflavine up to 1000 μg./ml. We have therefore confined the results we present to those derived either from spread plates or from gradient plates.

RESULTS

Fluctuation tests

Two separate experiments were carried out, exactly according to the method of Luria & Delbrück (1943). Calculations from the results showed in each instance a variance in numbers of resistant organisms in the separate cultures which was significantly greater than that expected by random distribution ($P < 0{\cdot}1$). Nevertheless, the variance was not large and we felt it might possibly have been due to environmental factors.

Indirect selection by replica plating

Isolation of proflavine-resistant strains. The proflavine-resistant mutants obtained by replica plating were named M1 to M5 in the order of their isolation. The method of isolation is summarized in Table 1.

Table 1. *Summary of replica-plating experiments for isolation of proflavine-resistant mutants of* Escherichia coli

Selection	No. of cycles of replication and type of inoculum*	Incubation period of master-plate (hr.)	Subcultures from master-plate	Proflavine conc. used (μg./ml.)
S36 ⟶ M1	4 (C, C, S, S)	24	Broth or slopes	5–50
S36 ⟶ M2, M3	7 (C, C, X, S, X, X, S)	24 (5 for last)	Direct†	10, 20, 50
M2 ⟶ M4	4 (C, C, X, S)	5	Direct (broth for last)	100, 150
M4 ⟶ M5	4 (C, C, X, S)	5	Direct	400, 500

* C = crowded plate, 10^6–10^7 organisms; X = criss-cross streaks; S = single colony.
† Direct on to next master plate for subsequent replica plating.

*M*1. This was derived from sensitive strain 36 by a series of 4 replica plating operations, with plates containing 5–50 μg. proflavine/ml., and with an intermediate subculture in broth between sets of replica plating. It was regarded as a first-step mutant, and repeated attempts were made to isolate a second-step mutant from it. They were all unsuccessful, although more resistant strains could be easily obtained by direct selection from proflavine plates.

*M*2. Another first-step mutant was obtained from strain 36, 7 sets of replicas being necessary on plates containing proflavine 10, 20 and 50 μg./ml. From the final set, two colonies M2 and M3 were picked off, which originally had different colony forms. They were found to have exactly the same resistance, and the difference in colony form disappeared after a few subcultures. We therefore retained only M2 for further work.

*M*4. A master plate was made from M2. Concentrations of proflavine 100–150 μg./ml. were used for making the replicas. Four replications yielded a pure culture of strain M4.

*M*5. Here M4 was used to make the master plate, and the drug plates contained proflavine 400 and 500 μg./ml. Four sets of replicas were needed to give a pure culture of strain M5. Two attempts to obtain a yet more resistant strain from M5 were unsuccessful.

Isolation of chloramphenicol-resistant mutants. From the sensitive strain 36, 2 first-step mutants were isolated, L1 and L2. L2 was slightly more resistant than L1. The concentrations of chloramphenicol used for the replica plates were 2, 3 and 4 μg./ml.

The resistance of first-step mutants

Both M1 and M2 were included in this category, since the chance of picking up a double mutant during replica plating is remote. Strain M2 corresponded in resistance to most of the strains previously obtained from strain 36 plated on proflavine-containing agar (see Thornley & Yudkin, 1959). The pattern observed here differed from that described by Cavalli & Maccacaro (1952) for chloramphenicol. These authors found first-step mutants which had many different degrees of resistance. The resistance shown by M1, on the other hand, was only once encountered amongst a large number of strains picked from colonies on proflavine agar. Thus, the concentration of proflavine which decreased the count to 1 in 1000 was 55 μg./ml. for strain M1, and 82 μg./ml. for strain M2. It was thought probable that this unusual strain was isolated in our earlier attempts at replica plating because of its exceptionally good growth in competition with other strains. When mixtures of two strains were subcultured 14 times in broth, M1 held its own with its parent strain 36 and overgrew a more resistant strain obtained by 'training'. At this stage, we had not isolated other first-step mutants by replica plating, so that we did not test the relative growth of M1 against these. If, however, as seems likely from these earlier results, M1 was unusually vigorous, the intermediate broth culture which we were then using between sets of replicas could well have resulted in overgrowth of second-step mutants by this strain. This would account for our failure to isolate second-step mutants from M1.

Step-wise mutation to proflavine resistance

The strains M2, M4 and M5 were regarded as a series representing first-, second- and third-step mutants to proflavine resistance. Resistance to different drug concentrations was determined by spread plate counts for the sensitive strain and for each of the mutant strains (Figs. 1 and 2; Table 2).

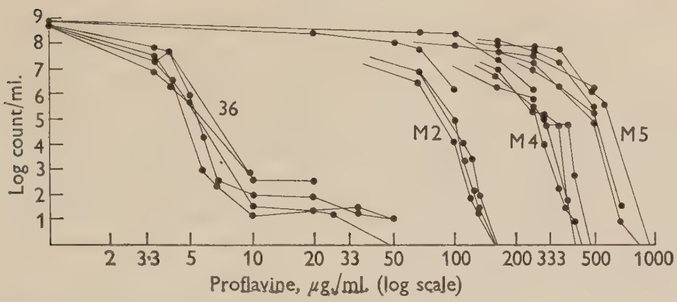


Fig. 1. Resistance of sensitive *Escherichia coli* 36 and of mutants. The curves represent counts on spread plates containing varying amounts of proflavine. Each curve represents one determination of resistance; the determination was repeated on different occasions. 36 = sensitive organisms; M2 = first-step mutant, from 36; M4 = second-step mutant, from M2; M5 = third-step mutant, from M4.

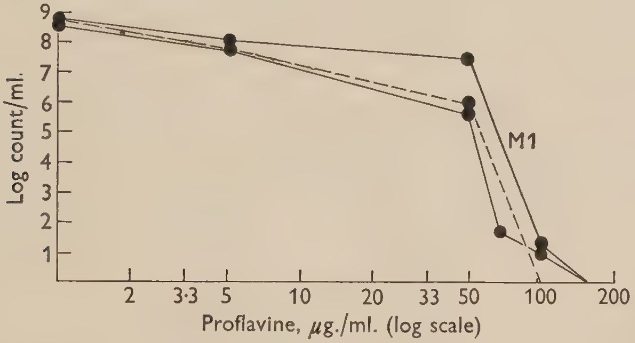


Fig. 2. Resistance of first-step mutant M1, from sensitive *Escherichia coli* 36.

Table 2. *Resistance of sensitive and mutant strains of* Escherichia coli 36

(Proflavine concentration giving a survival of 10^{-2} and 10^{-3} of the original organisms. Calculated by interpolation from Fig. 1.)

Survival	Strain 36	Strain M2	Strain M4	Strain M5
	Proflavine concentration, log μg./ml.			
10^{-2}	0·602 ± 0·031	1·873 ± 0·040	2·330 ± 0·028	2·532 ± 0·028
10^{-3}	0·665 ± 0·023	1·917 ± 0·032	2·416 ± 0·025	2·655 ± 0·028

The exact resistance of each strain and the exact differences between strains varied slightly according to the method of measurement. If we take the criterion of resistance as the concentration of drug which decreases the number of colonies to 1 in 1000 on spread plates, then the relative resistance of the strains may be expressed thus:

$$36 \xrightarrow{\times 18} M2 \xrightarrow{\times 3{\cdot}2} M4 \xrightarrow{\times 1{\cdot}8} M5.$$

The strain of highest resistance, M5, is approximately 100 times more resistant than sensitive strain 36. If we use the criterion of the diminution in colony count to 1 in 100, the increase between strains 36 and M5 is 85 times.

First-step mutants to chloramphenicol resistance

The resistance of the two chloramphenicol-resistant mutant strains L1 and L2 was compared with that of the sensitive strain 36 by growth on plates with increasing concentrations of chloramphenicol, and by gradient plates. The factor of increased chloramphenicol resistance was 7 for strain L1 and 10 for strain L2. Spread plate counts with strains 36 and L1 also gave a factor of about 7 for the ratio of chloramphenicol concentrations needed to reduce the colony count by 99%.

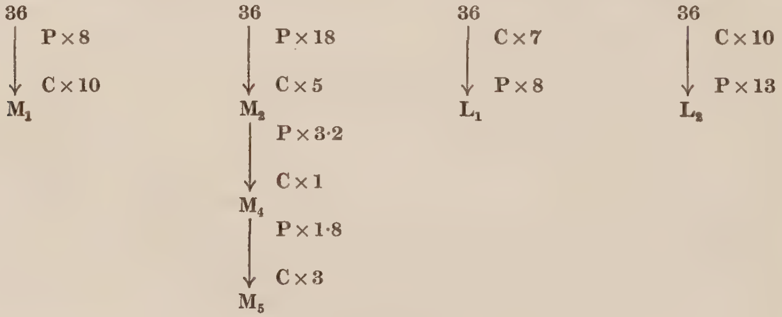
Cross resistance of mutants to proflavine and chloramphenicol

The 4 mutants isolated for resistance to proflavine, and the 2 for resistance to chloramphenicol, were each tested on gradient plates containing the other drug. Table 3 shows the increase in resistance between successive mutants, both to proflavine and to chloramphenicol, the number indicating the factor by which the resistance increases.

Table 3. *Cross-resistance of mutants to proflavine and chloramphenicol*

36 = original sensitive strain *Escherichia coli* 36.

Increase in resistance from one step to the next shown thus: P × 8, i.e. proflavine resistance increased eightfold; C × 10, i.e. chloramphenicol resistance increased tenfold.



DISCUSSION

The final procedure which we adopted for indirect selection by replica plating was found to be most effective and enabled a series of 4 or 5 replica platings to be carried out in 1 week. The omission of intermediate culture on broth was intended to eliminate the period of competitive growth between sensitive and mutant organisms. It is well known that mutants often tend to grow more slowly than the wild type in the absence of drug. Strain M1 was unusual in being able to persist through many subcultures in the presence of the sensitive strain 36. It is noteworthy that this strain M1 was the only one isolated in the early stages of this work, when broth subcultures were being used. A period of incubation of the master plate, shorter than the usual 18 or 24 hr., was suggested by Lederberg as one of the possible variations of the technique, and has been used by several other workers (e.g. Sneath, 1955). It has the obvious advantage of better resolution on the replica plates, since the very small colonies will not spread sideways. Also, in the early stages of the process, where a large inoculum is used, there is less chance of resistant colonies being covered over by surrounding sensitive organisms during the growth period. There is another advantage in the final stages of the enrichment process, when a master plate with isolated colonies is used. If such a colony is developing from a sensitive organism, and a mutation to resistance occurs during its growth, the final colony will consist of a mixture of sensitive and resistant organisms. It will therefore give colonies on the replica plates. Nevertheless, on subcultures from the master plate, predominantly sensitive organisms will be found if they have a growth advantage over the mutants. The use of a period of 5 hr. for incubation of the master plate, with a maximum colony size of about 1000 organisms, very much decreases the chance of mutations occurring during colony growth. That is, the colony which gives replicas on the drug plate is more likely to have arisen from a single mutant organism.

In practice, results which could be explained by this process were encountered during the isolation of M2 from 36. In one of the later sets of replicas, 3 master plates were inoculated, each with about 200 organisms, incubated for 24 hr. and replicated. Seven colonies on the master plates were thought to be resistant judging by the colonies on the drug plates, and inocula were picked from these seven positions, in some cases in duplicate, giving a total of 12 subcultures. All proved to consist mainly of sensitive organisms, some with a small proportion of resistant ones. Since using the 5 hr.-incubation period, we have always found that colonies corresponding in position to those on drug plates consisted only of resistant organisms. Lam & Sevag (1955) do not seem to have considered this possibility. They picked streptomycin-resistant colonies from the drug plates of a replica series, and the colonies in corresponding positions on the master plate, and they found a much higher resistance in the colonies obtained from the drug plates. This is exactly as would be expected if a mutation had occurred during colony growth on the master plate. These authors, however, interpreted their results as evidence that contact with the drug was necessary to develop resistance.

It seems certain from our results that proflavine resistance in *Escherichia coli* may be acquired by a series of mutational steps, since increased resistance acquired in the complete absence of drug may be demonstrated by replica-plating. The same is true of the acquisition of at least a low degree of chloramphenicol resistance.

Of the 6 sets of mutants studied, 5 had increased resistance both to chloramphenicol and to proflavine. The sixth (from M2 to M4) showed an increase in proflavine resistance only. It would thus seem that the property of proflavine resistance may result from at least two different mechanisms of resistance. One would appear to affect resistance to both drugs, and the other resistance to proflavine only. This situation is similar to that observed by Cavalli (1952) for cross-resistance to chloramphenicol and terramycin.

We are grateful to the Medical Research Council for a personal grant to one of us (M.J.T.).

REFERENCES

CAVALLI, L. L. (1952). Genetic analysis of drug-resistance. *Bull. Wld Hlth Org.* **6**, 185.

CAVALLI, L. L. & MACCACARO, G. A. (1952). Polygenic inheritance of drug-resistance in the bacterium *Escherichia coli*. *Heredity*, **6**, 311.

DEAN, A. C. R. & HINSHELWOOD, C. N. (1952). The resistance of *Bact. lactis aerogenes* to proflavine (2:8-diaminoacridine). I. The applicability of the statistical fluctuation test. *Proc. Roy. Soc.* B, **139**, 236.

ERIKSEN, K. R. (1949). On the validity of the fluctuation test in studies on the mode of origin of penicillin resistant staphylococci. *Acta path. microbiol. scand.* **26**, 269.

LAM, G. T. & SEVAG, M. G. (1955). Mechanism of the development of resistance to streptomycin. II. Biochemical differences of replica colonies. *J. Bact.* **69**, 184.

LEDERBERG, J. & LEDERBERG, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.

LURIA, S. E. & DELBRÜCK, M. (1943). Mutation of bacteria from virus sensitivity to virus resistant. *Genetics*, **28**, 491.

SNEATH, P. H. A. (1955). Proof of the spontaneity of a mutation to penicillinase production in *Bacillus cereus*. *J. gen. Microbiol.* **13**, 561.

THORNLEY, M. J. & YUDKIN, J. (1959). The origin of bacterial resistance to proflavine. 1. Training and reversion in *Escherichia coli*. *J. gen. Microbiol.* **20**, 355.

(*Received* 21 *October* 1958)

Sinai, J. & Yudkin, J. (1959). *J. gen. Microbiol.* **20**, 373–383

The Origin of Bacterial Resistance to Proflavine

3. The Alleged Rapid Adaptation to Proflavine Resistance in *Bacterium lactis aerogenes* (syn. *Aerobacter aerogenes, Klebsiella pneumoniae*)

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SUMMARY: It was reported (Baskett, 1952) that small additions of proflavine made at intervals to a growing culture of *Bacterium lactis aerogenes* (synonyms *Aerobacter aerogenes, Klebsiella pneumoniae*), did not prevent continued growth; this was regarded as proof that drug resistance might be acquired by adaptation. This phenomenon does not occur with *Escherichia coli* grown in broth, but we confirm that it occurs with *B. lactis aerogenes* grown in a chemically defined medium containing glucose. Growth of the aerogenes organism ceased when all the proflavine was added at once. Examination of the aerogenes organisms at the end of the experiments showed that a high proportion had become slightly more resistant, and a few had become highly resistant. These changes were not sufficient to account for continued growth of the aerogenes organisms in increasing concentrations of proflavine. In high concentrations of proflavine, growth of sensitive organisms did occur when some filtrate from a drug-free culture was added to the culture medium. It also occurred when the growing culture was kept acid, or when the medium was made acid before inoculation. On the other hand, growth did not occur in the presence of proflavine when the culture was kept neutral. It is concluded that the growth of *B. lacis aerogenes* in cultures to which proflavine is added gradually is chiefly due to a decrease of the inhibitory action of the drug by the acid formed during growth in a glucose + salts medium. The absence of the phenomenon when *E. coli* was grown in broth can be explained by the absence of glucose so that there was no lowering of pH value. There is thus no evidence of a rapid adaptation to proflavine resistance in *B. lactis aerogenes*.

One of the mechanisms suggested for the origin of drug resistance is a phenotypic adaptation induced by the drug (e.g. Hinshelwood, 1949; Dean & Hinshelwood, 1953). However, the evidence presented by Hinshelwood and his colleagues, as well as by other workers, is mostly inconclusive, since the experimental conditions do not eliminate the possibility of mutation and selection. There is, however, a publication by Baskett (1952) from Hinshelwood's laboratory, which seemed to prove that *Bacterium lactis aerogenes* (synonyms *Aerobacter aerogenes, Klebsiella pneumoniae*) may become resistant to proflavine by direct induction. Baskett added proflavine in small amounts at intervals to a rapidly growing culture. He found that the culture was still able to grow, though rather more slowly than usual, even when quite high concentrations of drug had been achieved. The rate of growth in presence of the drug depended on the original rate of growth of the culture and on the rate of addition of proflavine. Large inocula transferred to new media containing the drug were able to resume this growth. Small inocula, however, were not able to grow in a new medium containing the same high concentration of drug as had been achieved in the experiment. Baskett explained this phenomenon by assuming a rapid adaptation of the organisms, the rate of this adaptation being represented by the maximal rate at which proflavine could be added. That these adapted organisms did not grow when inoculated in small numbers into fresh medium containing proflavine was explained by Baskett by the short time of adaptation, which had consequently not been able to 'impress' itself sufficiently on the organisms to become permanent. The obvious explanation of these results is the change induced in the organisms by the drug, acting in these conditions, so that they become able to grow in concentrations far higher than would permit growth of the original sensitive organisms. This explanation has been accepted by a number of observers (e.g. Schnitzer & Grunberg, 1957). It was clearly important to confirm these experiments. It occurred to us, however, that a confirmation of the phenomenon did not imply a confirmation of Baskett's explanation. Briefly, one can consider that the growth of the organisms in the conditions of the experiment might be due either to changes in the organisms so that they become more resistant, or to changes in the medium so that the inhibitory action of proflavine is decreased.

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The experiments to be described were designed to test these possibilities. They fell into three groups: (1) to see whether the phenomenon occurs with *Escherichia coli* and to confirm that it occurs with *Bacterium lactis aerogenes*; (2) to determine whether the organisms show an increased resistance and if so something of its extent and persistence; (3) to determine whether the medium shows changes which decrease the inhibitory effect of proflavin and if so to discover their nature.

METHODS

Organisms. The strain of *Bacterium lactis aerogenes* was that used by Hinshelwood and colleagues, and was a culture kindly supplied by Sir Cyril Hinshelwood.

A culture of *Escherichia coli* NCTC 8196 from the National Collection of Type Cultures was also used in some experiments. *E. coli* 36 was mainly used, which had been isolated in this laboratory from a single colony of *E. coli* NCTC 8196 (see Thornley & Yudkin, 1959).

Media. The chemically defined medium containing glucose and ammonium sulphate was used for *Bacterium lactis aerogenes* as described by Baskett (1952). The components were (g./l.) glucose, 20; disodium hydrogen phosphate, 6·16; potassium dihydrogen phosphate, 1·14; ammonium sulphate, 0·96; $MgSO_4.5H_2O$, 0·038.

Nutrient broth (Difco) was used for experiments with *Escherichia coli.*

Nutrient agar (Difco) was used with both organisms for counts, sterility tests and drug gradient plates.

Proflavine hemisulphate (*British Drug Houses Ltd.*) was sterilized dry by heating for 1 hr. at 100°. A fresh stock solution was made up in the appropriate sterile medium for each experiment.

Growth of organisms. The procedure was designed to allow for the inoculation of cultures into the medium, for the aeration of the medium kept at constant temperature, for the addition of solutions of proflavine, and for the withdrawal of samples. The experiments were carried out in 250 ml. Buchner flasks containing 50 ml. medium. The inoculum was 0·5 ml. of an overnight culture of organism in nutrient broth. The flasks were kept in a bath at 40° for *Bacterium lactis aerogenes* or at 37° for *Escherichia coli.* Aeration was effected through a manifold by a compressor. Additions were made and samples withdrawn under sterile conditions through a syringe to which was attached a narrow tube of polythene.

Measurements of relative growth were made by measuring changes in optical density with a Spekker spectrophotometer. The use of filter H508 eliminated changes in optical density through changes in proflavine concentration. The curve of readings plotted against dilution was linear up to an optical density of 0·4; densities above this were determined by previous dilution with medium.

Additions of proflavine. The solution of proflavine was added always at intervals of 10 min. to give concentrations in the medium increasing at each addition by 5 μg./ml. for *Bacterium lactis aerogenes* and by 2 μg./ml. for *Escherichia coli.*

Measurement of relative growth was made by optical density and expressed as $\log_2$ Spekker reading. In this way, 1 unit = 1 cell division.

Culture filtrates were prepared usually after 5 hr., i.e. at the end of the logarithmic growth phase. The samples were centrifuged for 30 min. at 1500 rev./min. and then filtered through sintered glass of porosity 5. The filtrates were usually kept in a refrigerator overnight before being used in the experiments.

pH *values.* Preliminary readings were made by British Drug Houses Ltd. universal indicator; more precise measurements were made with a pH meter.

Tests for contamination. At the end of all experiments cultures were routinely tested for contamination by plating on nutrient agar and staining by Gram's method.

RESULTS

Experiments were made to confirm the observations of Baskett (1952) with *Bacterium lactis aerogenes*, and also to see whether the phenomenon occurs with *Escherichia coli.*

Experiment 1. *Bacterium lactis aerogenes.* Four flasks of glucose ammonia medium were inoculated at the same time. They were treated as follows:

Flask 1. Two hr. after inoculation, when turbidity was just visible, additions of proflavine solutions were begun, and continued at 10 min. intervals until 24 additions had been made, i.e. for 4 hr. The final concentration of proflavine, allowing for dilution by the additions and for taking out of the samples, was 97 μg./ml.

Flask 2. Additions of proflavine were begun after 250 min. of incubation and were continued at 10 min. intervals for 4 hr.

Flask 3. After 250 min. of incubation without proflavine, the drug was added in one amount to give a final concentration of 97 μg./ml.

Flask 4. No addition of proflavine was made to this flask.

From all flasks, samples were withdrawn 2 hr. after inoculation, and thereafter at 2 hr. intervals.

The results are given in Fig. 1. Gradual addition of proflavine allowed the growth of *Bacterium lactis aerogenes*, the growth rate being quite high (Fig. 1, curves 1 and 2). The optical density of the culture at the beginning of proflavine addition affected the resulting growth rate; that is, when the optical density was low (curve 1), the growth rate was lower. Addition of the whole amount of proflavine inhibited the growth almost completely. These results confirm the observations of Baskett.

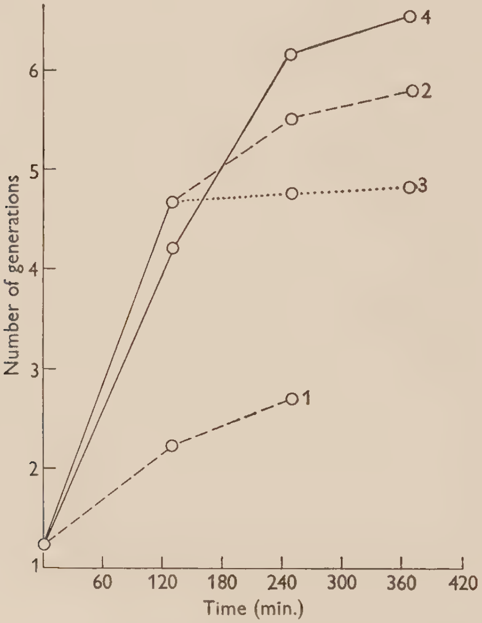

Fig. 1. Effect of proflavine on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, Proflavine added gradually from 120 min. after inoculation; 2, proflavine added gradually from 250 min. after inoculation; 3, proflavine all added at 250 min. after inoculation; 4, no proflavine added. – – – – = Proflavine added gradually; ——— = no proflavine added; $\cdots\cdots$ = proflavine added at once 97 μg./ml. Time from 120 min. after inoculation. Growth measured by turbidity.

Experiment 2. *Escherichia coli*. This was carried out in a similar manner to Expt. 1 but with *E. coli* in nutrient broth instead of *Bacterium lactis aerogenes* in glucose ammonia medium. Since *E. coli* is more sensitive to proflavine, lower concentrations of it were used, the final concentration being 20 μg./ml. Unlike the previous experiment, however, very little growth occurred after addition of proflavine, although the experiment was repeated several times with various modifications. Thus Baskett's phenomenon of 'rapid adaptation' did not occur with *E. coli* under these conditions.

Changes in resistance of organisms

These experiments were designed to determine what changes in resistance and morphology occur in the organisms during the Baskett 'adaptation'.

Experiment 3. *Changes in resistance.* The *Bacterium lactis aerogenes* organisms from Expt. 1 were streaked on to a gradient proflavine plate containing 100 μg./ml. (for details of this technique see Thornley & Yudkin, (1959). The bacteria from the control to which no proflavine had been added grew to the middle of the plate; this then represents the normal resistance of *B. lactis aerogenes.* The bacteria from the culture to which gradual additions of proflavine had been made showed a solid streak about 30% longer than the control; in addition, several colonies of higher resistance appeared up to the end of the plate. Bacteria from the culture to which the whole of the proflavine had been added at once did not grow at all on the gradient plate; a very few colonies grew on a drug-free plate. Thus, the high concentration of proflavine added at once rendered most of the organisms nonviable. Gradual additions, however, led to a slight increase in resistance in a high proportion of organisms and to the emergence of several highly resistant mutants. The slight increase in resistance, which incidentally might easily have been overlooked on ordinary serial proflavine plates, has implications which are discussed later. But neither this nor the emergence of the relatively few highly resistant mutants is able in itself to explain the high growth rate of the organisms which occurred in increasing concentrations of proflavine.

Experiment 4. *Changes in morphology.* At the beginning and end of Expt. 1, viable counts were made on the cultures which had been grown with and without proflavine. The increase in number of viable organisms was 50 times in the control without proflavine, as compared with twice in the presence of proflavine added gradually (flask 1). Gram-stained films of the cultures showed that the organisms growing in the presence of proflavine were mostly in the form of long filaments. Thus the addition of proflavine had inhibited division more than growth in mass.

Changes in medium

In this series of experiments we examined whether changes occur in the medium during the Baskett type of 'adaptation' which might enable the organisms to grow in the presence of proflavine.

Experiment 5. The purpose of this experiment was to see whether the interaction of a bacterial filtrate and proflavine, in the conditions of the Baskett experiments, resulted in the medium now being able to support the growth of sensitive organisms. The drug was added at intervals, at 40° with aeration, to a mixture of fresh medium + filtrate of a culture which had not been in contact with proflavine. At the same time, an inoculation of sensitive *Bacterium lactis aerogenes* organisms was made into fresh medium, and after 3·5 hr., the organisms from this culture were added to the proflavine medium and incubation continued. Parallel with this, the usual Baskett type of experiment was carried out. There were thus four flasks, as follows:

Flask 1. This contained 10 ml. fresh glucose ammonia medium + 40 ml. sterile filtrate from a 5 hr. culture of sensitive organisms in similar medium. Proflavine 5 μg./ml. was added every 10 min. at 40° for 3·5 hr. with constant aeration until the concentration reached 66 μg./ml. The medium was inoculated with packed washed *Bacterium lactis aerogenes* from 50 ml. of a culture of sensitive organisms in the middle of the logarithmic phase of growth. The incubation was then continued.

Flask 2. Glucose ammonia medium (50 ml.) was inoculated in the usual way with 0·5 ml. of an overnight culture of *Bacterium lactis aerogenes*. After 3·5 hr., proflavine was added in steps to a final concentration of 66 μg./ml.

Flask 3. This was set up as flask 2, but after 3·5 hr. all the proflavine was added at once.

Flask 4. This was set up as flask 2, but no proflavine was added.

The results are given in Fig. 2. The admixture of sterile filtrate of a sensitive culture (which was never in contact with proflavine), and proflavine added gradually at 40° with aeration, resulted in a medium that supported growth of *Bacterium lactis aerogenes* in a normally completely inhibitory proflavine concentration, i.e. 66 μg./ml. The growth rate increased during the experiment from 16 to 23% normal (flask 1).

In some experiments, we measured the pH value of the glucose ammonia medium during growth. We found a gradual decline from pH 7·2 to pH 4·0–4·5 in medium with or without proflavine. It seemed possible, therefore, that the antagonistic action of the culture filtrate on proflavine inhibition was due to its acidity.

Experiment 6. We therefore studied the effect of different pH values on growth in culture filtrate with proflavine. To four flasks was added filtrate from a 5 hr. culture of sensitive *Bacterium lactis aerogenes*. In three flasks, 4 parts of filtrate were diluted with 1 part of fresh medium; the fourth contained undiluted filtrate. The pH value of 2 flasks was adjusted with dilute NaOH to pH 6·8 and 6·0; the third had a pH of 5·5 and the fourth (undiluted filtrate) a pH value of 4·0. All flasks contained a total volume of 50 ml. of diluted or undiluted filtrate. Proflavine was added gradually in the usual way to a final concentration of 61 μg./ml. At this stage, packed washed *B. lactis aerogenes* from a 3·5 hr. culture in 50 ml. glucose ammonia medium was added to each flask, and incubation with aeration continued.

The results are illustrated in Fig. 3. At pH 6·8 and 4·0, growth did not occur in filtrate + proflavine. At pH 6·0 and 5·5, growth occurred at 12–14% of the normal rate. Leaving aside for a moment the fact that growth did not occur at pH 4 in the undiluted medium, these results confirm that the interaction of filtrate and proflavine, which decreased the inhibitory action of the drug, occurred only when the filtrate was acid and was abolished when the acidity was neutralized. The absence of this effect at pH 4 may be due either to the pH value being too low, or to the fact that no fresh medium was added.

It was observed that there was a coarse red precipitate in this flask at the end of the experiment.

Experiment 7. The possibility that the culture filtrate contained a proflavine-inactivating enzyme was investigated in this experiment. As part of Expt. 6, some of the diluted filtrate of pH 5·5 was boiled for 10 min. The proflavine was added at intervals in the usual way. After 2 hr., with proflavine at 61 μg./ml., an inoculum of packed *Bacterium lactis aerogenes* was added and incubation continued for 90 min. Growth occurred in the boiled and unboiled filtrate at the same rate of 14 % normal. Although it is possible that a heat-resistant enzyme was present in culture filtrates, the more likely explanation is that the effect was not due to an enzyme.

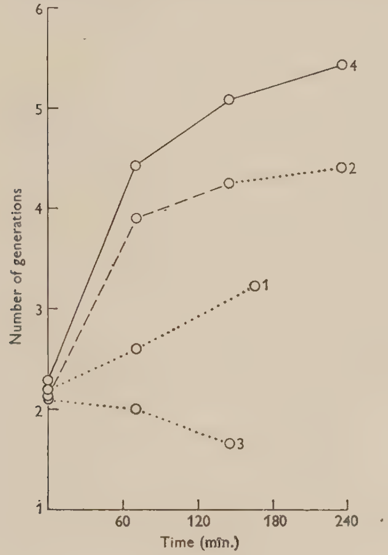

Fig. 2

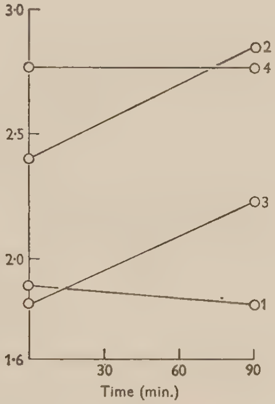

Fig. 3

Fig. 2. Effect of bacterial filtrate from a sensitive culture in glucose ammonia medium on the inhibitory action of proflavine on *Bacterium lactis aerogenes*. The relative amounts of growth were measured by turbidity. 1, Filtrate 40 ml. + fresh medium 10 ml., to which proflavine was added gradually then inoculated with washed logarithmic phase organisms; 2, proflavine added gradually from 210 min. after inoculation; 3, all proflavine added at 210 min. after inoculation; 4, no proflavine added. – – – – = gradual proflavine addition; ······ = 66 μg. proflavine/ml. present; ——— = no proflavine added. Time from addition of organisms in (1) and from 210 min. after inoculation in others.

Fig. 3. Effect of varying pH of bacterial filtrate on the inhibitory action of proflavine on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, Filtrate diluted by fresh medium 4:1, pH 6·8; 2, filtrate diluted by fresh medium 4:1, pH 6·0; 3, filtrate diluted by fresh medium 4:1, pH 5·5; 4, filtrate undiluted, pH 4·0. Gradual proflavine additions to final concentration of 61 μg./ml., then inoculated with washed organisms 210 min. after inoculation. Time was measured from addition of organisms to proflavine + filtrate; amount of growth was measured by turbidity.

Experiment 8. We attempted to prove that the phenomenon of growth in increasing concentrations of proflavine was due to the antagonistic action of a low pH value on the inhibition by proflavine. If this were so, two consequences follow. First, if the pH were kept neutral during the gradual addition of proflavine, no growth should occur. Secondly, if the pH of the culture were lowered at the beginning of the experiment, then the addition of all the proflavine at once should not stop growth; it would do so in the usual Baskett experiment because insufficient acid had at this time been produced. In a

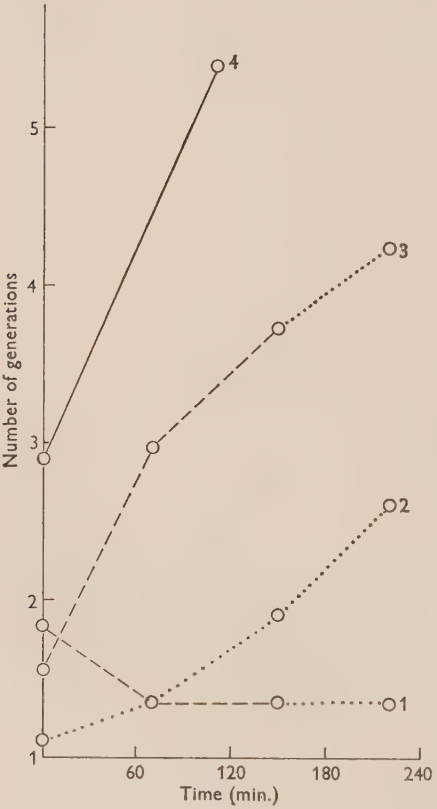

Fig. 4

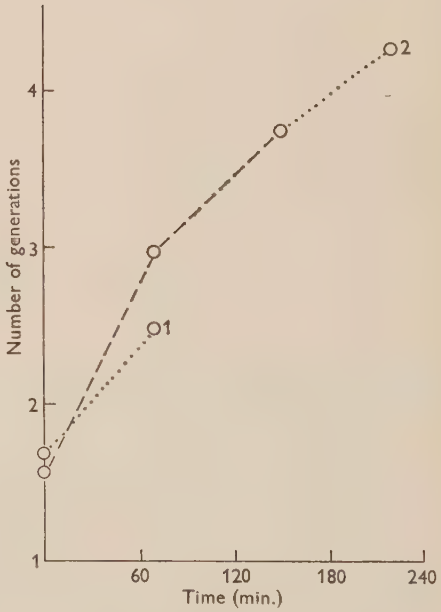

Fig. 5

Fig. 4. Effect of maintaining neutral pH in culture during proflavine additions, and of acidifying the culture with large single addition of proflavine made on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, pH maintained neutral throughout incubation, proflavine added gradually; 2, culture acidified to pH 4·9, then all proflavine added at once; 3, proflavine added gradually; 4, no proflavine added. – – – – = gradual proflavine additions; ······ = 65 μg. proflavine/ml. present; ——— = no proflavine added. Time was measured from beginning of proflavine additions; growth was measured by turbidity.

Fig. 5. Effect of acidifying medium before gradual proflavine additions on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, Medium acidified to pH 5·5, proflavine added gradually, then inoculated with washed packed organisms; 2, gradual proflavine additions to culture in logarithmic phase. – – – – = Gradual proflavine additions; ······ = 65 μg. proflavine/ml. present. Time was measured from inoculation in flask 1, and from beginning of proflavine additions in flask 2; growth was measured by turbidity.

series of preliminary experiments, we measured with a pH meter the changes in pH of a growing culture with and without gradual additions of proflavine. As well as this we measured the effect on growth of the addition of NaOH at intervals in amounts needed to restore the pH value to 7·2.

Experiment 8. A flask was set up in the usual way, for the gradual addition of proflavine to a culture of *Bacterium lactis aerogenes* in the logarithmic phase. Before the beginning of the addition, and throughout the addition, the pH was kept constant at 7·0–7·2 by the addition of NaOH. No growth occurred, and there was a decrease in turbidity (Fig. 4).

The pH of a growing culture was adjusted to 4·9 just before the addition of proflavine in one amount to a concentration of 65 μg./ml.; incubation was then continued. Growth occurred, the rate increasing during incubation from 10 to 30 % normal and the pH declining further to 4·2. In other flasks, whose pH values were not adjusted, the pH decreased during incubation, with or without proflavine to about 4·5 roughly in proportion to bacterial density. The results (Fig. 4) therefore confirmed the prediction made on the basis that the growth of the culture during addition of proflavine was due to the antagonism of the inhibitory action of proflavine by an acid medium.

Experiment 9. Fresh glucose ammonia medium was adjusted to pH 5·5. Proflavine was added gradually to 65 μg./ml. This acid proflavine medium was then inoculated with packed *Bacterium lactis aerogenes* from a 3·5 hr. culture and incubated with aeration for 90 min. The experiment was controlled by a flask submitted to the usual Baskett procedure, that is, a flask in which proflavine was gradually added to a growing culture.

The medium acidified to pH 5·5 supported growth in presence of 65 μg. proflavine/ml.; the growth rate was 27 % normal (Fig. 5). *Bacterium lactis aerogenes* was thus able to grow in fresh glucose ammonia medium in the presence of high concentrations of proflavine, provided the pH was low enough. This low pH value is that which is reached in experiments like those of Baskett in which continuous growth was achieved by gradual additions of proflavine which eventually reached the same final high concentration.

DISCUSSION

Our experiments confirm that *Bacterium lactis aerogenes* is able to grow in high proflavine concentration while this drug is added gradually to a culture growing in glucose ammonia medium. The explanation, however, is not, as Baskett (1952) suggested, that the organisms adapt themselves to the drug, but that during growth in presence and absence of proflavine the pH value of the culture drops from neutral to about 4·5–5·0. Low pH values antagonize the inhibitory action of proflavine (Peacocke & Hinshelwood, 1948; Albert *et al.* 1945).

Let us now consider Baskett's experiment in the light of our findings. Baskett started adding proflavine gradually to a culture in logarithmic phase (already turbid). In this the pH value was already sufficiently low to enable the organisms to tolerate 5 μg. proflavine/ml. Growth therefore continued, the pH value was decreased again and this in turn enabled the organisms to tolerate 10 μg. proflavine/ml. Further growth led to further decrease in pH value, and to increasing tolerance to proflavine. When Baskett added all the proflavine at once, no growth occurred because at the beginning of the logarithmic phase the pH value was not sufficiently small to enable this large amount of proflavine to be tolerated.

The ability to grow in high proflavine concentration is not inheritable. Baskett found that when he subcultured a small proportion of the 'adapted' cells into proflavine medium they did not grow. But when he subcultured a high proportion (dilution about 1/3), growth continued at the same high drug concentration. This is understandable when we remember that with this inoculum he lowered the pH value in the fresh proflavine medium. Here also the increased number of mutants, and the slight increase in resistance that we found, might play a role.

We have proved in several ways that there is no phenotypic adaptation in the Baskett phenomenon. The sterile filtrate of a 5 hr. culture of sensitive organisms, which were never in contact with proflavine, supported growth of *Bacterium lactis aerogenes* in the presence of the same high proflavine concentration and at a similar rate as in the usual Baskett type of experiment. This is the most important evidence against adaptation because in this filtrate there were no products of 'adapted' cells. The main factor which antagonizes the proflavine action in this filtrate is its low pH value. This was supported by two further observations. When the pH value was maintained at neutral during the usual Baskett experiment, no growth occurred, and when the pH value was decreased to that achieved at the end of Baskett's experiment and the whole amount of proflavine was added at once, growth occurred at a similar rate to that in the Baskett type of experiment. Finally, the low pH value itself is sufficient to explain Baskett's results, because growth occurred when fresh medium was acidified to pH 4·9, proflavine added and then sensitive organisms inoculated into it.

Although the decrease in pH value is sufficient to explain the Baskett phenomenon, it seems likely that other factors contribute to the actual rate of growth achieved in the presence of proflavine. These include: selection of mutants of high resistance; slight increase in resistance in most of the organisms; the products of metabolism (other than H ions) of a growing culture which might supply metabolic intermediates (Albert, 1951).

There still remains to mention the different rates of growth in our experiments. The growth rates in the Baskett type of experiment varied from 5 to 21 % of normal at 65 μg. proflavine/ml., and were somehow dependent on the bacterial density at which the proflavine additions started. In our experiment with filtrates supporting growth, the growth rates varied with different pH values of filtrates and dilution by fresh medium, the maximum at 65 μg. proflavine/ml. being 14–23 % of normal. In a culture acidified to pH 4·9 and with all proflavine added at once, the rate was 10 %, increasing to 30 %; in the acidified medium it was 27 %. We consider these are in reasonable agreement with the values found by Baskett.

The probable reason why *Escherichia coli* growing in broth did not grow in the Baskett type of experiment is that the pH value did not decrease, owing to the absence of glucose. We conclude that the experiments of Baskett, which appeared to give the strongest support to Hinshelwood's theory, do not in fact support it. The fact that, in producing proflavine resistance, phenotypic adaptation does not occur rapidly and to a high degree does not, however, exclude the possibility that it may occur more slowly or to a lower degree. This possibility is investigated in the following paper.

REFERENCES

ALBERT, A. (1951). *The Acridines.* London: Arnold and Co.

ALBERT, A., RUBBO, S. D., GOLDACRE, R. J., DAVEY, M. & STONE, J. (1945). The influence of chemical constitution on antibacterial activity. Part II. A general survey of the acridine series. *Brit. J. exp. Path.* **26**, 160.

BASKETT, A. C. (1952). The resistance of *Bact. lactis aerogenes* to proflavine (2:8-diaminoacridine). II. The direct induction of adaptation. *Proc. Roy. Soc.* B, **139**, 251.

DEAN, A. C. R. & HINSHELWOOD, C. N. (1953). Observations on bacterial adaptation. In *Adaptation of Micro-organisms. Symp. Soc. gen. Microbiol.* **3**, 21.

HINSHELWOOD, C. N. (1949). Adaptation of bacteria to resist drug action (with special reference to *Bact. lactis aerogenes*). *Symp. Soc. exp. Biol.* **3**, 243.

PEACOCKE, A. R. & HINSHELWOOD, C. N. (1948). The absorption of antibacterial substances (2:8-diaminoacridine and methylene-blue) by cells of *Bact. lactis aerogenes. J. chem. Soc.* p. 2290.

SCHNITZER, R. J. & GRUNBERG, E. (1957). *Drug Resistance of Microorganisms.* New York: Academic Press Inc.

THORNLEY, M. J. & YUDKIN, J. (1959). The origin of bacterial resistance to proflavine. 1. Training and reversion in *Escherichia coli. J. gen. Microbiol.* **20**, 355.

(*Received* 21 *October* 1958)

SINAI, J. & YUDKIN, J. (1959). *J. gen. Microbiol.* **20**, 384–399

The Origin of Bacterial Resistance to Proflavine

4. Cycles of Resistance in *Escherichia coli* and their Bearing on Variations in Resistance in Cultures

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SUMMARY: Small amounts of proflavine were added at intervals of 30 min. to growing cultures of *Escherichia coli*. Additions during the logarithmic phase gave an increase of resistance of two- or threefold in a high proportion of organisms. Additions in the lag or late logarithmic phase gave no such increase, although the resistance of the organisms themselves was higher in these phases. Whether organisms were able to grow in the presence of proflavine, therefore, depended not only on their resistance but on the conditions in the culture medium. The increase in proflavine resistance, which occurred when drug was added to growing cultures, was not accompanied by increase of cross-resistance to other drugs. The resistance was lost on growth in the absence of drug. For these reasons, the increase is held to be a phenotypic adaptation. There was also an increase in the number of organisms with a high resistance, of the order of that of mutants. These organisms showed cross resistance with other drugs.

Partial synchronization of division was achieved by the temporary cooling of cultures. These synchronized cultures showed cycles of division of about 30 min. They also showed cycles of resistance of *c.* 20 min. Evidence is presented for the view that the organisms undergo cycles of varying adaptability to proflavine resistance. It is suggested that this variation in adaptability can explain the range of resistance found in an ordinary sensitive culture. It can also explain the effect of proflavine additions in raising the resistance of a high proportion of organisms in a growing culture.

Our experiments with '*Bacterium lactis aerogenes*' showed that rapid adaptation to high degrees of proflavine resistance did not occur (Sinai & Yudkin, 1959). There was, however, some small increase in resistance in the presence of the drug. In the present paper we describe experiments which demonstrate that, in appropriate conditions, a similar small increase in resistance occurs with *Escherichia coli*. The results have a bearing on the variation in resistance found in the organisms of a sensitive culture. This variation is seen for example in the usual 'survivor' curves where most organisms are found to be prevented from forming colonies over a fairly narrow range of drug concentration, but some are inhibited by lower concentrations and some only by higher concentrations (Fig. 1; see also Thornley & Yudkin (1959), Figs. 1 and 2).

There is at present no explanation of this phenomenon, which has experimental backing. The proponents of the 'mutation-selection' theory of the origin of drug resistance only speak of 'phenotypic variation' (Cavalli, 1952). The proponents of the adaptation theory speak of two opposing forces, the lethal action of the drug and the adaptation of the organism towards resistance. Neither attempt an explanation of why there should be any variation between organisms in either their sensitivity or in their ability to adapt. The theory of clonal variation (Yudkin, 1953) suggests that the variation in resistance in a culture arises from asymmetrical division, but whilst there is evidence that this occurs (Hughes, 1952, 1955), it has not been proved that it is responsible for the continuous curve of variation of resistance in a culture. A possible explanation might lie in a variation in resistance with physiological age of the cells, i.e. from division of the parent cell to division of the daughter cells. Since a growing culture would have organisms of the whole range of physiological age, it would then have the whole range of resistance.

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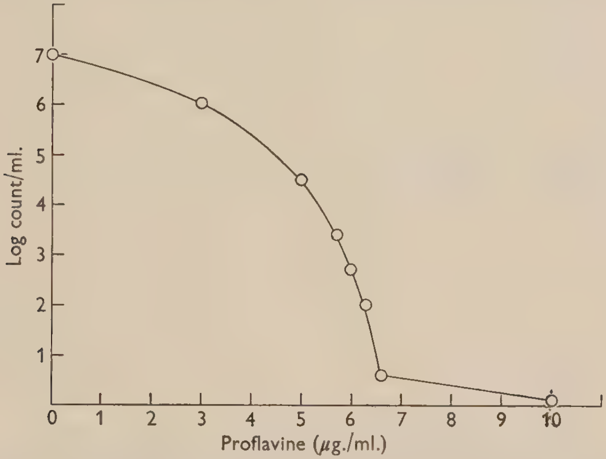

Fig. 1. **Distribution of resistance in a sensitive culture of *Escherichia coli* 36. Number of colonies which appear on plates with different concentrations of proflavine.**

We have shown that there is a number of mutational steps to increased proflavine resistance in *Escherichia coli* (Thornley & Yudkin, 1959). One can imagine that on this is superimposed a continuous variation of resistance of a narrower range for both the sensitive organisms and for each of the mutations; this would give a much more continuous range of resistance. We confined ourselves therefore to a study of the range of resistance expressed in the normal sensitive culture, and assume that whatever we were able to demonstrate as to its origin probably applies to the mutants of successively higher degrees of resistance. The first series of experiments were designed chiefly to test whether it was possible to increase the resistance of a sensitive culture in a continuous manner. The second series of experiments tested the possibility that the variations in resistance within a culture were due to differences in physiological age of the bacteria constituting the culture.

In experiments on the effect of small additions of proflavine on resistance of a sensitive culture, proflavine was added at intervals to cultures of *Escherichia coli* at different periods of growth. The amounts added were such as not to inhibit growth completely. The intervals were roughly equal to the mean generation time. By this means, we sought in a relatively short time to produce a culture of significantly higher resistance than the original culture. It was of course necessary to ensure that such an increased resistance, when it occurred, was not due to a multiplication of pre-existing mutants.

We investigated: (i) The degree of increase of resistance. This was done usually by the gradient plate technique, which is very sensitive. Sometimes we also measured the number of organisms of increased resistance, by dilution counts on solidified medium containing proflavine. By 'increased resistance', we mean a resistance intermediate between that of the sensitive cells, and that of first-step mutants. (ii) The degree of inhibition by the drug. This was measured both as an inhibition of the increase of mass of organisms, and as an inhibition of the increase in number of viable organisms. (iii) The number of organisms of high resistance at the beginning and end of each experiment. These were assumed to be mutants. (iv) The stability of the organisms of increased resistance. (v) The resistance to other drugs (cross-resistance).

Experiments were also designed to produce cultures in which divisions were synchronized, and to test the resistance of the organisms at various times. By this means we hoped to detect changes in resistance in organisms of roughly the same physiological age during the cycle from one division to the next.

METHODS

Details of the organism *Escherichia coli* strain 36 and of the drugs are given in the previous papers in this series (Sinai & Yudkin, 1959; Thornley & Yudkin, 1959).

Growth of organisms. The cultures were made in 50 ml. nutrient broth held in 250 ml. Buchner flasks. The inoculum was 0·2 ml. over-night culture in nutrient broth. The flasks were kept in a water bath at 37°. In most instances the cultures were aerated by an air compressor through a manifold. A syringe with a polythene extension was used to take samples and to make drug additions, without disturbing the growing cultures. At the end of each experiment, routine checks were made that the cultures had remained neutral and that they were not contaminated by other organisms.

Measurements of growth. Growth in mass was estimated by the optical density as measured by the Spekker spectrophotometer. Growth in numbers of viable bacteria was measured in agar plates. Serial dilutions of the cultures were made and 0·5 ml. mixed with 9·5 ml. nutrient agar. Plates were poured in triplicate.

Measurement of drug resistance. This was done by dilution counts and by gradient plates (Szybalski, 1952). For dilution counts, proflavine pour-plates proved unsatisfactory since the resistance to proflavine is higher in semi-aerobic conditions. We therefore spread 0·1 ml. of cultures of various dilutions on the surface of a series of plates of solid medium containing proflavine at various concentrations. In different experiments there was appreciable variation in the number of resistant organisms surviving at given concentrations of proflavine. This seems to be due to slight differences in the medium which affected the inhibitory action of the low concentrations of proflavine which were being used. For this reason, we have only compared the results within each group of experiments in which the same batch of nutrient agar was used.

For measurements with gradient plates we were concerned to avoid the effect of differences in the gradients of drug concentration in different plates. We have therefore always compared the resistance of cultures from streaks on the same plates. If, then, the treated culture gave a streak on this plate 50 % longer than that given by the untreated culture, we call this an increase in resistance by a factor of 1·5.

Definitions of degrees of resistance. Our normal untreated culture of strain 36 was sensitive to about 10 μg. proflavine/ml., that is, only one or two organisms in 10^6 gave colonies in this concentration. These organisms proved to be the first-step mutants demonstrated by Thornley & Yudkin (1959). Up to a concentration of about 7 μg. proflavine/ml., there was a continuously decreasing number of organisms able to give colonies. In such a normal culture, then, the majority of organisms did not give colonies on, for example, 5 μg./ml. Sometimes after treatment a culture was able to give a much higher proportion of colonies at such a concentration of proflavine, but not necessarily a higher number at 10 μg./ml., at which first-step mutants did. We refer to such a culture as one of 'higher resistance'. In general then this culture had a resistance greater than that of the original culture but lower than that of a culture of first-step mutants.

Synchronization of division. We used the technique of temporary cooling described by Hotchkiss (1954) in his experiments on Pneumococcus. Cooling was carried out either rapidly or slowly. In the first method, the culture was placed in iced water until it reached the required temperature, and then in a water bath at that temperature. Slow cooling was carried out by placing the culture directly into a water bath at the required temperature. A variety of times and temperatures of cooling were used, and the cultures were of various ages. No improvement in synchronization was achieved by the second period of cooling. In appropriate conditions, quite good synchronization was achieved. An example of the results is given in Fig. 2. Here cultures were cooled at different intervals after inoculation. They were placed in iced water for 2 min., then held at 12° for 15 min., and finally returned to 37°. Counts were made before and immediately after cooling, and then at intervals of 10 min.

The best synchronization was achieved in this experiment when cooling began 70 min. after inoculation. On returning to 37°, there was a burst of divisions in the next 10 min., followed by a division cycle of 30 min. Half of the divisions occurred in the first 20 min., and the other half in the next 10 min. A second division cycle followed.

It may be added that we have frequently determined viable counts, also at 10 min. intervals, of cultures which had not been cooled. None of these experiments showed the step-wise increase in numbers shown, for example, in Fig. 2, B, C and Fig. 8, A.

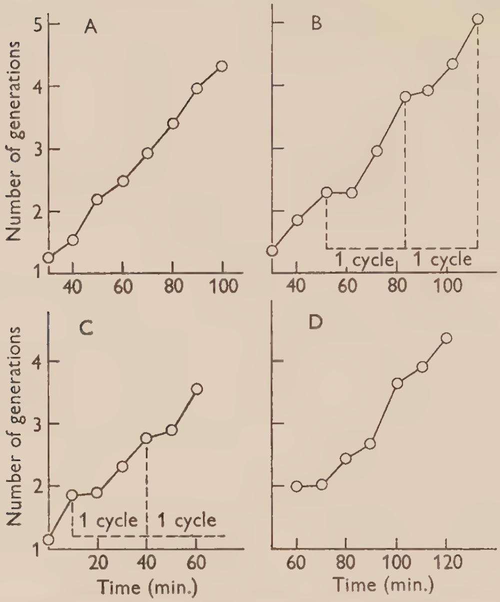

Fig. 2. Growth of a synchronized culture of *Escherichia coli* 36. Synchronization of division by cooling to 12° for 15 min., begun at different times after inoculation. Growth measured by viable counts. Cooling started at min. after inoculation: A, 120; B, 90; C, 70; D, 60. Time from returning the flasks to 37° after cooling.

RESULTS

Effect of small additions of proflavine on the resistance of a sensitive culture

Several experiments were performed in which proflavine was added in small amounts at 30 min. intervals to a growing sensitive culture. The first additions usually brought the concentration of proflavine to 1 μg./ml. Subsequent additions were constant in each experiment, but differed in different experiments from amounts which increased the concentration by 0·15 μg./ml. with each addition, to amounts which increased it by 0·4 μg./ml. We give details of one such experiment.

Experiment 1. Fifty ml. nutrient broth in a 250 ml. Buchner flask were inoculated with 0·2 ml. of a 17 hr. culture. After incubation for 2 hr., the culture was divided into two parts, and incubation continued. To one flask, proflavine was added to 1 μg./ml. At 30 min. intervals, further additions of proflavine were made, each increasing the concentration by 0·3 μg./ml., so that a final concentration of 3·7 μg./ml. was achieved after 270 min. The second flask had no added proflavine, and served as control. Samples were taken for measurements of mass growth by optical density, and for measurements of resistance. The latter was done by streaks on gradient plates containing 13 μg. proflavine/ml., and by counts of the number of organisms which gave colonies on plates containing proflavine at 6·6, 10 and 20 μg./ml. The results of this experiment are shown in Table 1. The results of a similar experiment are also shown in Fig. 3, in which proflavine was added in different amounts to two flasks.

Table 1 shows that by the end of the first experiment the resistance as measured on gradient plates increased by a factor of 3 (i.e. an increase of 200 %) when proflavine was added, and by a factor of 1·3 (an increase of 30 %) in the control culture without proflavine. This increased resistance was shown also by the number of organisms which gave colonies on plates with 6·6 μg. proflavine/ml. At the beginning of the experiment, there were 30 such colonies from 1 ml. of culture. At the end, the culture with proflavine gave 3200 normal sized colonies and 2×10^6 small colonies; the control culture gave 1000 normal sized colonies, but no small colonies.

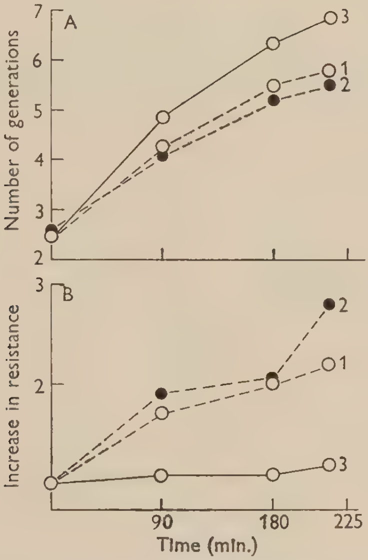

Fig. 3. Effect of addition of proflavine to a growing culture of *Escherichia coli* 36 on increase of resistance. Curve 1. Proflavine added gradually. First addition to 1 μg./ml., subsequent additions every 30 min. increasing concentration by 0·25 μg./ml. to final 2·75 μg./ml. Curve 2. Proflavine added gradually. First addition to 1 μg./ml., subsequent additions every 30 min. increasing concentration by 0·4 μg./ml. to final 3·8 μg./ml. Curve 3. No proflavine added. A, Growth, measured by turbidity; B, resistance measured by gradient plates. Time from 120 min. after inoculation.

The counts on the plates with 10 and 20 μg./ml. suggest that the organisms which gave these small colonies could not do so at the higher concentration of proflavine. The colonies which did appear at these concentrations were of normal size. We believe that these arose from the multiplication of pre-existing first-step mutants. Their number was about five times greater from the culture to which proflavine had been added from the control culture. This was probably due to an advantage in growth of the mutant organisms in the presence of proflavine.

The increased length of streaks on the gradient plate, and the appearance of large numbers of organisms which gave colonies on plates with 6·6 μg. proflavine/ml., showed that the addition of small amounts of proflavine to a growing culture of *Escherichia coli* in the logarithmic phase gave an increase in resistance to a degree below that of the first-step mutant. We believe, in fact, that the increased length of the streaks was caused chiefly, if not entirely, by the organisms with increased resistance.

Table 1. *Effect of addition of proflavine on resistance of* Escherichia coli 36

Comparison of increased resistance as measured on proflavine gradient plates, and by dilution counts on proflavine plates. Flask 1: gradual proflavine additions, to final concentration of 3·6 μg./ml. Flask 2: no proflavine added.

Flask	Time* (min.)	Proflavine present (μg./ml.)	Viable count, (colonies/ml.)	On proflavine (count/ml.)			Factor increase in resistance (gradient plates)
				6·6 μg./ml.	10 μg./ml.	20 μg./ml.	
1	0	0	3×10^7	3×10	<10	<10	—
	120	2	2×10^8	1×10^3 3×10^6 s	$3{\cdot}6 \times 10^2$	$2{\cdot}5 \times 10^2$	—
	270	3·6	1×10^8	$3{\cdot}2 \times 10^3$ 2×10^6 s	$1{\cdot}5 \times 10^3$	$1{\cdot}5 \times 10^3$	3
2	0	0	$2{\cdot}5 \times 10^7$	3×10	<10	<10	—
	120	0	$6{\cdot}4 \times 10^8$	$2{\cdot}5 \times 10^2$	$1{\cdot}5 \times 10^2$	$1{\cdot}4 \times 10^2$	—
	270	0	2×10^9	1×10^3	3×10^2	$2{\cdot}8 \times 10^2$	1·3

* Time from 135 min. after inoculation.
s = small colonies.

The inhibition of growth by the added proflavine was to some extent an inhibition of increase in bacterial mass and to a greater extent an inhibition of division. Thus this experiment showed that, between 120 min. and 270 min., there was a 21% inhibition of growth in mass, and a twofold decrease in viable count division; examination of smears showed many organisms forming filaments.

Experiment 2. Growth in this experiment was measured by optical density and by viable counts. Resistance was measured both by gradient plates with 14 μg. proflavine/ml. and by plates with proflavine 6·6, 10 and 20 μg./ml.

A culture 135 min. after inoculation was divided into two flasks. One acted as control. To the other was added 1 μg. proflavine/ml., and further 30 min. additions of 0·33 μg./ml. Samples were taken just before the first addition, after 2 hr. and after 4·5 hr.

In the control, the growth rate decreased after 4·25 hr. from inoculation. By 6·75 hr., the number of generations was 4·2 as judged by optical density, and 6·6 as judged by viable count.

In the flask with added proflavine, the number of generations at 6·75 hr. was 3·1 from optical density and 2·7 from viable count. In the period from 4·25 to 6·75 hr., the optical density increased twofold, but the viable count decreased. By the end of the experiment, the ratio of increase in mass to increase in number of viable organisms was 0·23 in the absence of proflavine and 2·7 in its presence. This suggests that proflavine inhibited cell division more than increase in mass. Microscopical examination showed that the organisms in the control culture became smaller during incubation, whilst those in the presence of proflavine became much larger.

Gradient plates showed again an increase of resistance by 30% in the absence of proflavine, and by 200% in its presence. On plates with 6·6 μg. proflavine/ml., the control culture gave about 1000 normal sized colonies/ml.; the culture with added proflavine gave 3000 such colonies, but also 2×10^6 small colonies. On plates with proflavine 10 or 20 μg./ml., only normal sized colonies appeared: about 250/ml. from the control culture and 1200/ml. from the culture with added proflavine. We shall refer later to the possible origin of the organisms which gave rise to small colonies on the proflavine plates.

Effect of physiological age of culture

Experiment 3. In this experiment eight cultures were used. To five of them proflavine additions were made in the way already described; the other three cultures acted as controls. The proflavine additions were begun at different times after inoculation, from 30 min to 5·25 hr. Thus they covered the range from the lag phase to the late logarithmic phase.

As in our earlier experiments, proflavine additions begun early in the logarithmic phase (flask 3) caused an immediate rise in resistance, as measured by streaks on gradient plates (Fig. 4). Proflavine added in the late logarithmic phase (flasks 4 and 5) did not cause any increase in resistance.

When proflavine additions were begun in the lag phase there was no increase in resistance for the first 4 hr. (flasks 1 and 2). After 4·25 hr. there was a rapid rise in resistance (flask 2). In flask 3, where additions were begun after 2·5 hr., there was an immediate rise in resistance. Thus by 4·5 hr. after inoculation there was an increase in resistance with additions begun at 2·5 hr., but not with additions begun at 45 min. At this time, however, there was about the same concentration of proflavine in both flasks, and the rate of growth of the culture was also very similar. Yet when, after the delay, the resistance began to rise in flask 2, it soon reached that in flask 3.

Again, as in previous experiments, those flasks which showed an increase in resistance measured by length of streaks on gradient plates also showed, when compared with the controls, about a fivefold increase in the number of organisms which gave rise to colonies on plates with 10 μg. proflavine/ml. Flasks 4 and 5, which showed no increase in resistance on gradient plates, also showed no relative increase in number of these organisms.

This experiment emphasizes that the ability of organisms to multiply in the presence of proflavine is due to a combination of two factors: the inherent resistance of the organisms, and the conditions in the medium. Thus organisms transferred from medium at the late logarithmic phase consistently gave streaks about 30% longer than those given by organisms in the lag phase. The organisms were therefore more resistant in the late logarithmic phase.

Yet proflavine added to cultures showed no inhibition of division in the lag phase, but considerable inhibition in the late logarithmic phase. Thus, in presence of their culture medium, the organisms showed a decreased resistance, though their inherent resistance was higher.

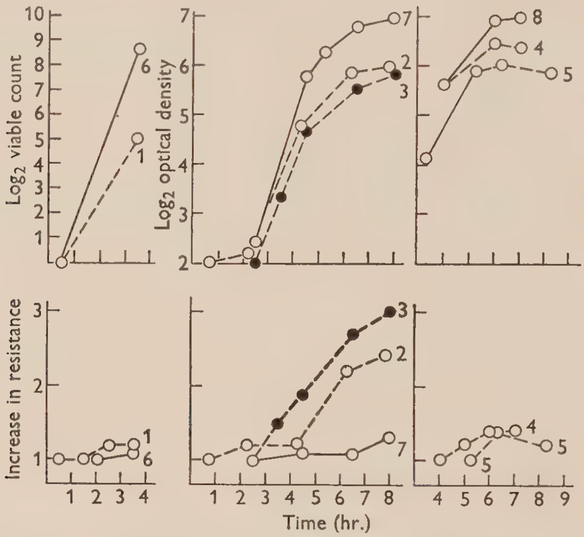

Fig. 4. Effect of addition of proflavine to cultures of *Escherichia coli* 36 at different phases of cultural growth, on increase of resistance as measured by gradient plates. Curve 1. Additions start in lag phase, 30 min. after inoculation. Curve 2. Additions start in lag phase, 45 min. after inoculation. Curve 3. Additions start in early logarithmic phase, 2·5 hr. after inoculation. Curve 4. Additions start in late logarithmic phase, 4 hr. after inoculation. Curve 5. Additions start in late logarithmic phase, 5·25 hr. after inoculation. Curve 6. Control for 1. Curve 7. Control for 2, 3, 4 and 5. Curve 8. Control for 4 and 5. ---- = Proflavine additions; ——— = no proflavine.

The stability of the increased resistance

Experiment 4. From several of the experiments where proflavine was added to growing cultures, samples of 0·05 ml. were inoculated into 5 ml. broth at the beginning and end of the experiment. After overnight incubation the resulting cultures were streaked on proflavine gradient plates. No difference in length of streak was found between cultures made at the beginning or end of the experiment, from either the control cultures or those with added proflavine. Thus, the increase in resistance found after the addition of proflavine to cultures in the logarithmic phase did not persist after subculture in the absence of drug.

At the end of one of the earlier experiments, inocula were taken from the beginning and from the end of the streak made on a gradient plate with 10 μg. proflavine/ml., both from the control culture and from the culture with added proflavine. In this experiment, the streak from the control culture showed during the experiment an increase in resistance by a factor of 1·2, and that from the culture with proflavine of 2·1. The inocula were streaked on to gradient plates with 20 μg. proflavine/ml.

Inocula from either end of the streaks made with the culture with added proflavine now gave streaks which grew right across the plate containing 20 μg. proflavine/ml. With the control culture there was an appreciable difference in the streaks made from inocula from the beginning (low proflavine concentration) and end (high proflavine concentration) of the original streak. Those from the beginning of the streak grew right across the plate. But those from the end of the streak gave only a short solid streak, and a few scattered colonies towards the end of high concentration.

A similar experiment was made with the small and the normal sized colonies which had appeared on a plate with 6·6 μg. proflavine/ml., with samples of the culture to which proflavine had been added. They were streaked on a gradient plate with 50 μg. proflavine/ml. The small colonies gave a thin dispersed growth across the plate; the normal sized colonies gave a thick solid growth across the plate.

The simplest explanation of these results is that small amounts of proflavine allowed an adaptation to a low degree of resistance. Unadapted organisms, from the control culture, can adapt to grow at a low concentration of the drug, but not to grow at a high concentration. Thus, the growth at the end of the streak represented organisms that were of poor viability, for when streaked again they give poor growth. Adapted organisms from the culture with proflavine can go on growing at a higher concentration. In either instance, the growth on the low or the high concentration in plates gave rise to a large number of highly resistant 'mutant' organisms within the colony. As would be expected, there were fewer of these in the small colonies than in the large, so that there was a difference in the streaks found on 50 μg. proflavine/ml. We believe that the organisms which gave small colonies on proflavine plates had become adapted in the presence of the drug and retained their resistance so long as the drug continued to be present. The increase in resistance which occurred when proflavine was added to growing cultures thus seems to be in the nature of an adaptation.

Cross-resistance

Experiment 5. We found that mutants selected by replica plating for resistance to proflavine were also resistant to chloramphenicol, Erythromycin, Aureomycin and Terramycin. The resistance to proflavine and to these drugs of a series of cultures to which proflavine had been added in the manner described in earlier experiments was tested on gradient plates with proflavine 10 μg./ml., chloramphenicol 4 μg./ml., Erythromycin 12 μg./ml., Aureomycin 75 μg./ml. or Terramycin 15 μg./ml. As in earlier experiments the gradual addition of proflavine increased the length of streak on proflavine plates, up to a maximum of three times the original length. The length of the streaks on the other drugs, however, fell. There was a rough proportionality between the increase in resistance to proflavine and the decrease in resistance to the other drugs. Together with the rapid loss of resistance on subcultivation in the absence of proflavine, we take these findings to support the view that the increased proflavine resistance when proflavine was added to growing cultures was different from the mutational change which gave proflavine resistance in cultures isolated by replica plating.

Proflavine resistance in relation to division cycles; cycles of resistance in a sensitive culture

Experiments were made to test the hypothesis that the continuous range of resistance in a sensitive culture, in which there were organisms of every stage of the division cycle, was due to differences in resistance at different stages of the division cycle. If there be such a cycle of resistance, two consequences follow which should be demonstrable in a synchronized culture. First, the proportion of organisms which survive at a given concentration of proflavine (i.e. the proportion of resistant organisms) should vary during the division cycle. Secondly, the period out of the whole division cycle during which organisms will be able to survive a given concentration of proflavine should be inversely proportional to that concentration. We should find, that is, that organisms from a synchronized culture are able to grow in relatively high concentrations of drug during only a small part of the cycle; they should grow in lower concentrations during a greater part of the cycle. The general plan of the experiments was to synchronize a culture by cooling (see Methods). After returning the flasks to 37°, samples were withdrawn at intervals of 5 or 10 min. and measured for total viable count and for the number of colonies which would grow on plates with one or more concentrations of proflavine.

Experiments 6–8. The results of three experiments are shown in Figs. 5–7. In general, we found cycles of division lasting 30 min., and cycles of resistance varying, in different experiments, from 15 to 30 min. Thus, Fig. 5 shows two division cycles, in each of which half the divisions occurred in the first 20 min. and half in the next 10 min. There were four complete cycles of resistance, each of 20 min. duration. During part of each resistance cycle, there were 10^5 or more organisms/ml. which formed normal sized colonies on 5 μg. proflavine/ml., and during the rest of the cycle about 10^3 such organisms. Fig. 6 shows that a lower degree of synchronized division, compared with Figs. 5 and 7, was associated with less pronounced and less regular cycles of resistance. After three or four resistance cycles, there was a gradual decrease in the degree of synchronization and of cycles of resistance.

We have no explanation for the difference between the periodicity of cycles of resistance and of division. Hotchkiss (1954) found a similar difference with Pneumococcus, with division cycles of 30 min. and transforming cycles of 40 min.

In conformity with our expectation, the amplitude of the cycles of resistance depended on the degree of resistance, i.e. on the concentration of proflavine used to test it. Thus Fig. 6 shows that there was no detectable periodicity in the number of organisms forming colonies on 4 μg. proflavine/ml., whilst the number which formed colonies at 5·5 μg./ml. showed a clear periodicity. The maximum variation of numbers here was by a factor of nearly 1000. Again, as predicted, the duration of the peak of resistance was shorter with higher concentrations of proflavine (Figs. 6, 7). Occasionally, though not always, the same number of organisms occurred at the peaks of the cycles when different degrees of resistance were being measured. Thus, in Fig. 7, this occurred for resistance to proflavine at 5 μg./ml. and at 5·9 μg./ml.

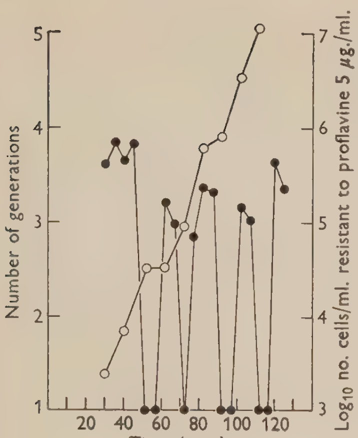

Fig. 5

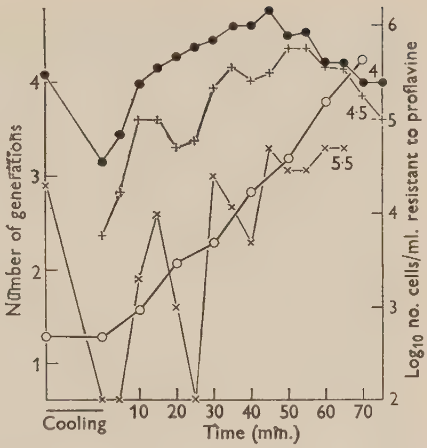

Fig. 6

Fig. 5. Changes in number of resistant organisms within the division cycles in a synchronized culture of *Escherichia coli* 36. Synchronization was obtained by cooling a culture after 90 min. from inoculation at 12° for 15 min. ○ = Total viable count expressed as number of generations; ● = number of organisms/ml. forming colonies on agar containing 5 μg. proflavine/ml., expressed as $\log_{10}$. Time from returning to 37° at end of cooling.

Fig. 6. Changes in number of resistant organisms within the division cycles in a synchronized culture of *Escherichia coli* 36. Synchronization was obtained by cooling a culture after 70 min. from inoculation at 17° for 15 min. Number of generations derived from viable counts. Number of organisms forming colonies on proflavine agar. ○—○ = Total viable count; ●—● = resistant to 4 μg. proflavine/ml.; +—+ = resistant to 4·5 μg. proflavine/ml.; ×—× = resistant to 5·5 μg. proflavine/ml. Time measured from end of cooling.

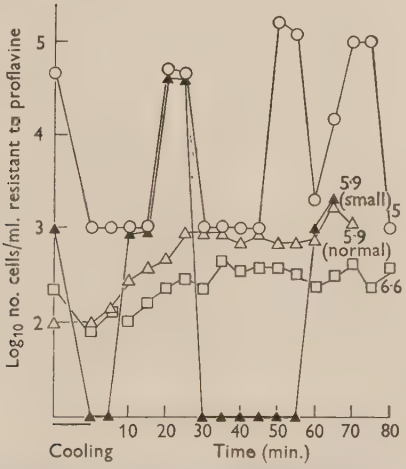

Fig. 7. Changes in number of organisms resistant to proflavine (μg./ml.) 5, 5·9, 6·6; in a partly synchronized culture within the division cycles. Synchronization was obtained by cooling a culture 90 min. from inoculation at 12° for 5 min. and then at 17° for 20 min. ○—○ = Resistant to 5 μg. proflavine/ml.; △—△ = resistant to 5·9 μg. proflavine/ml.: normal sized colonies; ▲—▲ = resistant to 5·9 μg. proflavine/ml.: small colonies; □—□ = resistant to 6·6 μg. proflavine/ml. Note: at 5 μg./ml. and 6·6 μg./ml., only normal-sized colonies occurred. Time from end of cooling.

Cooling did not affect the viability of the organisms but it did affect the degree of resistance. Immediately after cooling there was a decrease in the number of resistant organisms proportional to the degree of resistance tested. This is seen in Fig. 6, where there is a decrease of 1 log. unit in the number of organisms resistant to 4 μg. proflavine/ml. and of 3 log. units in those resistant to 5·5 μg./ml.

The presumed first-step mutants differed in two ways from the rest of the organisms in synchronized cultures. First, there was no change in the number of organisms resistant to, for example, 6·6 μg. proflavine/ml. after cooling. Secondly, there was no cycle of resistance, for example, in the number of organisms resistant to 6·6 μg./ml. Thus we believe that, in Fig. 7, the normal sized colonies formed on 5·9 μg. proflavine/ml., and the colonies on 6·6 μg./ml. which were all normal in size, were formed from organisms presumed to be first-step mutants. These findings were confirmed in a pure culture of the first-step mutant. Although synchronized division was produced there were no cycles of resistance when the culture was tested at a concentration of 10 μg. proflavine/ml. Control cultures which were not cooled for synchronization showed neither cycles of growth nor cycles of resistance (Fig. 8).

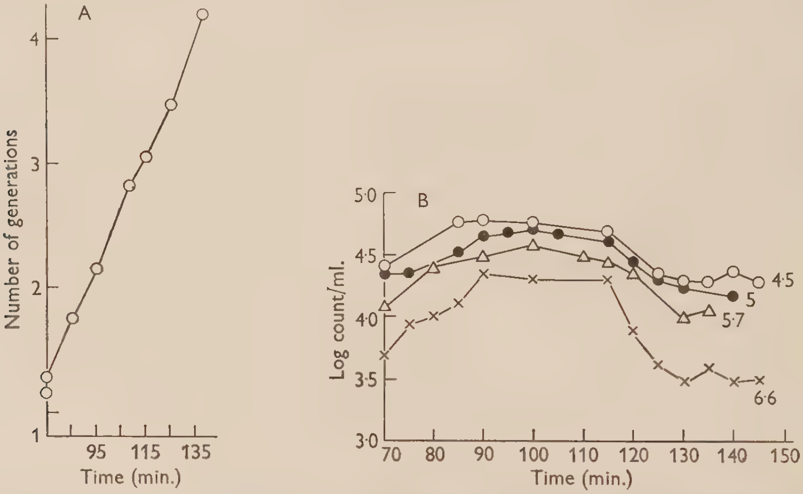

Fig. 8. Growth and resistance of non-synchronized cultures of *Escherichia coli* 36. A, Number of generations derived from viable counts. Time from inoculation. B, Resistance measured by number of organisms forming colonies on proflavine agar. Time from inoculation. ○—○ = Resistant to 4·5 μg. proflavine/ml.; ●—● = resistant to 5 μg. proflavine/ml.; △—△ = resistant to 5·7 μg. proflavine/ml.; ×—× = resistant to 6·6 μg. proflavine/ml.

DISCUSSION

The degree of synchronization we achieved was not complete and was rather less than that achieved with Pneumococcus by Hotchkiss (1954), who also used the cooling technique. On the other hand, it was about the same as that achieved by Maruyama & Yanagita (1956) with *Escherichia coli*, although they used a mechanical method less likely to injure the organisms. It is possible therefore that *E. coli* cannot be made to divide entirely synchronously. One puzzling fact was the difference between the period of the division cycles (about 30 min.) and the period of resistant cycles, usually 20 min., occasionally 15 or up to 35 min. Hotchkiss (1954) also found a difference, although in his experiments the cycles of transformability were always longer than the cycles of division. One possible explanation is that cooling inhibited more than one reaction concerned with division.

An important conclusion may be derived from a consideration of the experimental results and especially of the techniques used to demonstrate varying resistance during the division cycles. This is, that it is not the resistance of the organism which varies during the cycle, but its ability to remain resistant, i.e. its adaptability. Let us imagine the fate of an organism placed on solid medium containing drug. If this is done when the 'resistance' is low, it does not form a colony. When done when the 'resistance' is high, it does form a colony. But in so doing, it must have divided and passed through a period of a division cycle when, in the absence of proflavine, its resistance would have been low. Nevertheless, it was able to grow, repeatedly divide, and eventually give a colony in which the parent organisms would normally many times have passed through periods of low resistance. The only reasonable explanation of this is that it is the adaptability, not the resistance, of the organism which varies with the cycle; once an organism has adapted to increased resistance in the presence of the drug, it retains this resistance through all stages of the cycle, so long as the drug continues to be present.

The results give some information about the range of the cyclical variation in adaptable resistance, which can be compared to the resistance of first-step mutants. Throughout the whole, or most, of the cycle the organisms of a sensitive culture are able to form colonies on plates with 4 μg. proflavine/ml. We may then say that this concentration or a trifle less represents the inherent resistance of the organisms. On plates containing more proflavine, only organisms plated out at one part of the cycle can grow, and the higher the concentration of drug, the shorter is the period during the cycle when the organisms can go on growing. The maximal concentration of drug which seems to allow the organisms to grow during the part of the cycle when they had the highest degree of adaptability was about 7 μg. proflavine/ml. Above this concentration, only first-step mutants will grow. These seem to have an inherent resistance to about 10 μg. proflavine/ml. These mutant organisms show no fluctuation in sensitivity during the division cycle, in the same sort of way that the sensitive organisms show no fluctuation in the numbers at a concentration of 4 μg. proflavine/ml.

We also found that, immediately after cooling, the organisms seemed to have minimal adaptability to resistance. Thus at this stage only a small proportion of the sensitive organisms gave colonies on a drug concentration higher than about 5 μg./ml. whilst the unsynchronized culture before cooling contained a higher proportion of organisms which were able to do so. We have therefore two methods of distinguishing organisms at the stage of highest adaptability during the division cycle from those which pre-exist as mutants. First, the number of the former but not of the latter, which can grow on intermediate concentrations of drug, decreased immediately after cooling. Secondly, the former but not the latter show during the division cycle fluctuations in the numbers which are resistant to these intermediate concentrations.

We can now explain some of the results obtained when proflavine was added to growing (unsynchronized) cultures of *Escherichia coli*. Some of the organisms in these cultures would have been in the non-adaptable stage of the division cycle, and these would not then have continued to grow in the presence of the added proflavine. There is thus, as we found, a partial inhibition in growth. Other organisms would have been in the adaptable stage of the cycle, and so would adapt and continue to grow and divide. Transference from these liquid cultures on to plates with proflavine allows the formation of colonies from adapted cells. Transference on to media without proflavine results in a loss of the adaptation. Whilst therefore the increased resistance continues for as long as the organisms or their progeny are in contact with proflavine, it is lost in its absence. It is this last fact which suggests that the type of resistance with which we are here concerned conforms to the usual conception of phenotypic adaptation. This is supported by a lack of the cross-resistance shown by mutant organisms.

As to the degree of the phenotypic increase in resistance, we have seen that this is a function of the concentration of drug achieved during additions to the growing cultures. It would then seem possible to continue the additions so as to achieve a high degree of resistance. However, the possibility of killing the organisms limits the rate at which the drug can be added in the logarithmic phase. Moreover, the additions cannot be continued to give a high concentration because before this the culture enters the stationary phase when the organisms become sensitive to the drug. The appropriate conditions might be achieved in a culture kept continuously in the logarithmic phase in a chemostat, and such experiments would be worth doing.

In the conditions of some of our experiments, it seems that many of the organisms had become adapted so that they grew, though not readily, on a concentration of about 6 μg. proflavine/ml. The result was a smaller colony than usual. This we believe to be the explanation of the small colonies we found on such concentrations of proflavine.

The continuous curve of resistance found in an ordinary sensitive culture is, we suggest, mainly an expression of the variable adaptability of organisms at different stages of their division cycle. That is, since an ordinary culture comprises organisms of all stages of the division cycles, there is a continuous range of adaptability which is measured as a continuous range of resistance. It is this, or chiefly this, which, in the mutation-selection theory, is referred to as a phenotypic variation of a fixed genotype. We have not ruled out the possibility of a clonal variation due to asymmetrical division. If this exists, it would be a mechanism of continuous variation superimposed on that of a variable phenotypic adaptation in the division cycle. We believe that the degree of phenotypic variation is probably governed by the genotype. In our experiments, we have demonstrated a maximal adaptability to a resistance below that of the first-step mutant. It seems likely that in certain conditions the range may extend to or beyond that of the next genotype. It is possible that this and the succeeding steps of mutations have similar ranges of phenotypic adaptability. We suppose that there is in fact, in this and in other examples of multi-step mutation, a continuous range of resistance, or rather of phenotypic adaptability, bridging the intervals between the levels representing inherent genotypic resistance, from the most sensitive organism to the most highly resistant.

As well as the slight increase in resistance of a large proportion of the organisms, there was a considerable increase in the number of organisms able to grow on 10 μg. proflavine/ml. We have referred to these as if they were first-step mutants. It is, however, possible that some of these organisms are produced by an adaptation but of a higher degree and of a permanent nature.

Our results have a bearing on those described by Hughes (1952, 1955). He described slight differences in resistance of sister organisms separated after two or three divisions from a single organism. One possible explanation of these differences is that the organisms whose resistance was being measured were tested at different stages of the cycles of resistance.

REFERENCES

CAVALLI, L. L. (1952). Genetic analysis of drug-resistance. *Bull. Wld Hlth Org.* 6, 185.

HOTCHKISS, R. D. (1954). Cyclical behaviour in pneumococcal growth and transformability occasioned by environmental changes. *Proc. nat. Acad. Sci., Wash.* **40**, 49.

HUGHES, W. H. (1952). Variation in penicillin resistance in single-cell cultures of *Staphylococcus aureus. J. gen. Microbiol.* 6, 175.

HUGHES, W. H. (1955). The differences in antibiotic sensitivity of closely related single cells of *Proteus vulgaris. J. gen. Microbiol.* **12**, 269.

MARUYAMA, Y. & YANAGITA, T. (1956). Physical methods for obtaining synchronous culture of *Escherichia coli. J. Bact.* **71**, 542.

SINAI, J. & YUDKIN, J. (1959). The origin of bacterial resistance to proflavine. 3. The alleged rapid adaptation to proflavine resistance in *Bacterium lactis aerogenes* (syn. *Aerobacter aerogenes, Klebsiella pneumoniae*). *J. gen. Microbiol.* **20**, 373.

SZYBALSKI, W. (1952). Gradient plate technique for study of bacterial resistance. *Science*, **116**, 46.

THORNLEY, M. J. & YUDKIN, J. (1959). The origin of bacterial resistance to proflavine. 2. Spontaneous mutation to proflavine resistance in *Escherichia coli. J. gen. Microbiol.* **20**, 365.

YUDKIN, J. (1953). Origin of acquired drug resistance in bacteria. *Nature, Lond.* **171**, 541.

(*Received* 21 *October* 1958)

SINAI, J. & YUDKIN, J. (1959). *J. gen. Microbiol.* **20**, 400–413

The Origin of Bacterial Resistance to Proflavine

5. Transformation of Proflavine Resistance in *Escherichia coli*

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SUMMARY: We attempted to transform proflavine-sensitive strains of *Escherichia coli* to proflavine resistance by growth in the presence of deoxyribonucleic acid-containing extracts from resistant organisms. Three methods were used to obtain the DNA preparations. Method 1 (Boivin, 1947) did not give active transforming principle, even when a variety of modifications was introduced. Method 2 (McCarty & Avery, 1946) and Method 3 (Mayers & Spizizen, 1954) gave extracts which were active in transformation. With DNA prepared by Method 2, an increase in the number of resistant organisms was found in one of four rough sensitive strains. We concluded that only a small proportion of the organisms of this strain were competent. We were able to increase the proportion of these competent organisms by a method of 'double replica plating'. According to Method 3, organisms were lysed by sodium dodecylsulphate (Duponol) in presence of citrate, and protein removed by sodium acetate. From the supernatant fluid transforming principle was precipitated by acidified ethanol, and then dissolved in saline. The smooth strain of *Escherichia coli* used in most of our experiments served as the recipient strain. The transforming principle from resistant organisms was not active alone, but was active in the presence of the protein precipitate. The activity appeared to be lost when the transforming principle was treated with DNAase. No activity was shown by extracts from sensitive organisms. The activation of transforming principle by the protein precipitate is thought to be due to the Duponol carried with it. Duponol appears to inhibit DNAase, so that it might act by preserving transforming DNA from destruction by the enzyme present in the recipient organisms. Our experiments did not always give positive results. Whilst we believe that we have demonstrated transformation in this system, the low reproducibility of our results makes it necessary to repeat and extend.

Transformation may be defined as the acquisition by an organism from its environment, by non-sexual means, of a genetically active unit directing an inheritable change. The transforming principle behaves as a self-reproducing unit within the transformed organism (Austrian, 1952). The transformation phenomenon was first discovered by Griffith (1928) in Pneumococcus, and transformation by soluble transforming material in Pneumococcus has been demonstrated by McCarty & Avery (1946), Ephrussi-Taylor (1951) and Hotchkiss (1951); in *Haemophilus influenzae* by Alexander & Leidy (1950, 1951, 1953) and in *Neisseria meningitidis* by Alexander & Redman (1953).

There has, however, been no authenticated instance of transformation in *Escherichia coli*. Boivin (1947) claimed to have transformed *E. coli* from R to S. He reported success in only one out of 400 pairs of organisms which he tested. His effective strain has since been lost, and no further positive results have been reported.

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We were stimulated to study transformation of proflavine resistance of *Escherichia coli* for the following reasons. (1) In this species of organism, studies of recombination have revealed a structure analogous to chromosomes on which different determinants may be mapped. They suggest that the genetic activity of DNA may ultimately be linked to the organization of chromosomes. The fact that many mutants have been obtained from *E. coli* makes this species particularly useful in studies of the hereditary control of many characters. (2) We are mainly concerned in this work to show that several mechanisms for origin of drug resistance may operate for one system of bacterium and drug. To what extent transformation, if it occurs, may play a role in natural situations in causing the emergence of resistant strains will be discussed later. (3) The demonstration of transformation to proflavine resistance in *E. coli* would show conclusively that in this instance the change was genotypic.

METHODS

Organism. We worked with *Escherichia coli* No. 8196 obtained from the National Collection of Type Cultures (NCTC). *E. coli* strain 36 was a substrain from *E. coli* NCTC 8196 isolated in our laboratories. Strain 36 is a smooth strain, sensitive to 10 μg. proflavine/ml. Strains R1–R6 are rough substrains isolated from single colonies of strain 36. They were obtained by growing strain 36 in broth containing homologous rabbit antiserum, kindly prepared by Dr F. Himmelweit. They are all similar in morphology and their sensitivity on isolation was similar to that of the parent strain. Strain 22 is a highly resistant strain 'trained' by growing strain 36 on successively increasing concentrations of proflavine in nutrient broth. We assume that it is a multi-step mutant of the original strain. Strain M1 is a proflavine-resistant first step mutant isolated from strain 36 by the replica plating technique (Thornley & Yudkin, 1959). This means that it has never been in contact with the drug. Strain M4 is a second-step mutant isolated from strain 36 by the replica plating technique. This second-step mutant was isolated from a first-step mutant different from strain M1. It, too, was never in contact with the drug.

All strains were kept on nutrient agar slopes at 5° and transferred every 2 months.

Media and drugs were as given in our previous papers in this series.

Sodium dodecylsulphate (*Duponol*) was made up as 6% (w/v) solution in distilled water at pH 7 and freshly prepared for each experiment.

Sodium deoxycholate. A stock solution (2%, w/v) of this in distilled water was sterilized by incubation at 37° overnight and stored at 5°.

Assay of transformation of proflavine resistance

(1) *Bacterial counts.* Here we compared the ratio of the proflavine-resistant organisms to proflavine-sensitive organisms in transformation cultures and in control cultures.

(*a*) The total viable count was made on nutrient agar plates by spreading

0·1 ml. of serial dilutions. This enabled us also to test the purity of the culture and any changes in morphology of the organisms.

(*b*) Proflavine-resistant organisms were counted on nutrient agar plates containing different amounts of proflavine. Serial dilutions were made and 0·1 ml. spread on plates in duplicate.

(2) *Gradient plates.* The general change in the degree of resistance of the culture was assayed by streaking on gradient plates with different concentrations of drug. This method is described on p. 386 of this number. All plates were incubated at 37° for 24 hr.

Detection of DNA. This was done by the method of Stumpf (1947).

Nomenclature. Recipient organisms. Organisms which are grown in the presence of DNA extract. *Donor organisms.* Organisms from which DNA is extracted. *Transforming principle* (*TP*). This term is used for the DNA extract whether transformation is successful or not. Similarly, the terms 'transformation medium' and 'transformation culture' do not imply that transformation was necessarily successful.

RESULTS

Use of method of Boivin (1947)

By this means Boivin was able to transform a rough strain of *Escherichia coli* to smooth, though only a small proportion of his experiments were successful. We did the following experiments. Washed donor organisms were killed with toluene, the toluene removed by aeration, and the washed organisms suspended in 0·9 % saline. The suspension was heated to 100°, centrifuged and the deposit discarded. The supernatant fluid contained DNA as indicated by the Stumpf test; this was used as TP. Cultures of recipient organisms were made, diluted 1/10, and 0·1 ml. added to 4·5 ml. nutrient broth + 0·5 ml. TP. Controls which did not contain TP were also prepared. Five replicates were made of each mixture, and incubated for 48 hr. Samples of the replicates were pooled, and counts made of sensitive and resistant organisms after 24 and 48 hr. For donor organisms we used strains 22 and M1, and for recipient organisms we used strains 36, R1 and R2. We varied the age and density of the recipient organisms, the time of contact with the toluene, and the time and duration of extraction. In no instance did we observe an increase in the number of resistant organisms.

Use of method of McCarty & Avery (1946)

Our lack of success with experiments with TP prepared by the Boivin method might have been due to a destruction of the DNA by deoxyribonuclease (DNAase) extracted from the organisms at the same time. Perhaps, also, Boivin's success with only one pair of strains was due to an unusually low DNAase content of these strains. In the method which McCarty & Avery (1946) used for Pneumococcus, the DNA is protected from destruction by DNAase by inhibiting the action of the enzyme. The organisms are suspended in saline with sodium citrate, which forms a complex with the magnesium ions necessary for the action of DNAase. The organisms are then lysed by sodium deoxycholate, protein removed by chloroform-amyl alcohol and the DNA precipitated by ethanol. The DNA is dissolved in saline and kept at 5°. This method we applied as follows.

Cultures of *Escherichia coli* strain 22 (resistant) and strain 36 (sensitive) were grown in nutrient broth for 18 hr.; the organisms were centrifuged and washed with normal saline. They were then resuspended in a solution containing sodium deoxycholate 0·1% (w/v), sodium citrate 0·05 M, NaCl 0·1 M, and incubated for 3 hr. at 50°. This produced lysis of the organisms, although not so readily as with pneumococci, which McCarty & Avery found required only 1 hr. at room temperature. To the lysate was added 0·3 vol. of chloroform and 0·1 vol. of amyl alcohol, and the mixture shaken for 30 min. and centrifuged. A protein gel was formed at the interface, which was removed by pipetting. Ethanol (2 vol.) was added, when a granular and fibrous precipitate formed. The fibrous precipitate was lifted out with a glass rod and washed in ethanol. This TP was suspended in 5 ml. saline and stored at 5°.

The recipient organisms used were prepared from strains R1, R2, R3 and R4. A 3-hr. culture was made of each, diluted 1/10, and 0·1 ml. used for inoculation into transformation medium. This consisted of 4·5 ml. nutrient broth + 0·5 ml. TP preparation. The control medium for each culture consisted of 5 ml. nutrient broth. Each test and control culture was replicated five times and incubated for 18 hr. Bacterial counts on pooled samples of the five replicate tubes were made for total viable organisms and for organisms resistant to 10 μg. proflavine/ml.

Only one experiment showed a slight increase in resistant organisms by a factor of 4. This was strain R1 grown in TP prepared from the resistant strain 22. None of the other preparations showed any increase in number of resistant organisms.

This experiment suggested that the method of McCarty & Avery in the conditions used by us did not give active transforming principle. There was, however, a slight increase in resistance of one of the recipient strains, which might indicate the presence of a relatively few organisms in this strain which had competence to undergo transformation.

Isolation of a competent substrain

We tried to isolate a competent substrain from the *Escherichia coli* strain R1, which had shown a slight increase in resistance in the previous experiment. A culture was made of strain R1. After 18 hr. incubation, 0·1 ml. was spread on a nutrient agar plate and incubated for 6 hr. This master plate was replicated by velveteen mounted on a rubber bung on to a nutrient agar plate containing TP from resistant *E. coli*, together with 0·05 M-sodium citrate to inhibit the action of DNAase. The master plate was kept in the refrigerator, and the TP plate incubated for 24 hr. This was then replicated on to a plate containing 10 μg. proflavine ml., which was in turn incubated for 24 hr. At this time, 10 clusters of resistant colonies were detected on the proflavine plate. Subcultures were made from the corresponding areas on the master plate. The subcultures contained organisms which had not been in contact with TP or proflavine, and presumably contained either pre-existing proflavine-resistant mutants, or organisms which were capable of undergoing transformation to proflavine resistance. Their competence to undergo transformation was then tested.

TP was prepared from resistant strain 22 and from sensitive strain 36. Each of the ten cultures after incubation for 1 hr. was inoculated into medium containing 4·5 ml. nutrient broth + 0·5 ml. TP preparation, and also into control medium containing no TP. Incubation was continued for 18 hr. Bacterial counts were then made in the absence and in the presence of 10 μg. proflavine/ml. One of the ten strains tested showed an increased number of resistant organisms when incubated in presence of TP from the proflavine-resistant strain 22, but not when incubated with TP from the sensitive strain 36. The number of resistant organisms increased from 20 to 4000/ml. This apparently transformable strain we called *Escherichia coli* R1T. None of the remaining nine cultures showed this increase.

This experiment was repeated twice with successive broth cultures of strain R1T. Each time there was an increase in number of resistant organisms by 200 times when grown with extracts from resistant organisms, and no increase when grown with extracts from sensitive organisms. The transformed organisms retained this resistance when subcultured once in broth. After two further subcultures of R1T in broth, a repetition of the experiment gave a negative result.

That we were able three times to show an increased resistance in strain R1T when it was grown with extracts from resistant organisms, but not with extracts of sensitive organisms, suggests that we were dealing with an instance of true transformation. The apparent loss of competence of strain R1T on subculture may have been due to overgrowth with non-competent organisms, since we did not proceed further in our isolation than an area from the master plate which almost certainly consisted of mixed organisms. The results were nevertheless encouraging as a possible method of isolating competent organisms.

A further experiment was carried out with a different pair of organisms to see whether the phenomenon was more general. For a competent strain we chose strain R5; for the preparation of TP we used a proflavine-resistant mutant of strain M1, obtained by indirect selection. By replica plating as in the previous experiment, eleven areas were picked off from the master plate. Subcultures from them were tested with a preparation of TP from strain M1 and one from strain 36. An increase in number of resistant organisms occurred in one of the eleven strains when it was grown in the presence of TP from resistant organisms, but not when it was grown in TP from sensitive organisms. The increase in number of resistant organisms was of the order of 50 times. This strain we called *Escherichia coli* R5T. Like strain R1T, strain R5T seemed to lose its competence after a few subcultures in nutrient broth. We should stress here that, throughout the four years in which we have worked with this strain, we have never observed a significant increase in the number of resistant organisms in untreated cultures.

Use of method of Mayers & Spizizen (1954)

At this stage in our work, Mayers & Spizizen (1954) published their work on the preparation of active DNA from viruses and from *Escherichia coli*. The principle of this method is to dissociate the nucleo protein with a detergent and then to salt out the protein.

Preparation of TP. An 18 hr. culture of the organisms was centrifuged and washed once with saline containing 0·2 M-sodium citrate. To the packed organisms was added 6 % (w/v) sodium dodecylsulphate (Duponol) at pH 7. Lysis began immediately and was completed after stirring at 500 rev./min. for 45 min. A 3 M solution of Na acetate at pH 7 was added, equal to one-ninth of the original volume, the mixture warmed to 60° and stirred for 15 min. It was then cooled to 5° and centrifuged.

The supernatant fluid was added to 2·8 vol. acidified alcohol (95 %, v/v, ethanol, 5 %, v/v, methanol, with 1 part conc. HCl to 175 parts mixture). The strings of DNA were removed by a glass rod to a Buchner funnel and washed with 100 ml. ethanol. The DNA was placed in a centrifuge tube with some ethanol and left overnight at 5° to sterilize it. It was centrifuged and the deposit suspended in one-fortieth of the original volume of saline; it dissolved with great difficulty. It was stored at 5° and constituted our DNA preparation TP; it was Stumpf positive. This and all subsequent preparations of TP were routinely tested for sterility by plating on agar with and without proflavine.

The precipitate was washed twice with 3 M-sodium acetate, sterilized with ethanol as above and redissolved in saline to one-fortieth of the volume of the original culture. This protein fraction was also stored at 5° and constituted the protein preparation Pr.

Testing for transformation. A culture of *Escherichia coli* strain 36, as recipient strain, was taken 3 hr. after inoculation, and 0·1 ml. inoculated into four sets of tubes each in duplicate and each containing 4 ml. nutrient broth with: (i) 1 ml. saline (control); (ii) 0·5 ml. TP + 0·5 ml. saline; (iii) 0·5 ml. Pr + 0·5 ml. saline; (iv) 0·5 ml. TP + 0·5 ml. Pr. After incubation for 18 hr. counts were made on agar plates for total viable organisms and on proflavine plates for organisms resistant to proflavine.

Tests for transformation

Protein fraction and TP from resistant and sensitive organisms. The resistant organisms were from a culture of *Escherichia coli* M 4. This was a second-stage mutant isolated by replica plating (Thornley & Yudkin, 1959). Table 1 shows that the TP from the resistant organisms did not by itself increase the number of resistant organisms. The protein fraction alone increased the number of resistant organisms in 10^7 viable organisms from less than 10 to about 10,000 resistant to 20 μg. proflavine/ml., and from 0 to 500 organisms resistant to 50 μg. proflavine/ml. The colonies to which the organisms gave rise were small. The TP + protein fraction (PrM 4) together gave a 20 to 30-fold further increase in number of resistant organisms, and these gave rise to normal-sized colonies. The protein fraction from strain M 4, alone or with the TP, decreased the viable count to 10 % of that of the control.

Table 1. *Transformation of sensitive* Escherichia coli *strain 36 with extracts of resistant (mutant) organisms*

		Organisms/ml. resistant to proflavine (μg./ml.)		Organisms in 10^7 total viable organisms resistant to proflavine (μg./ml.)	
		20	50	20	50
Culture	Fraction present	No. org./ml.		No. org./10^7 viable	
1	—	$1{\cdot}5 \times 10^2$	0	7	0
2	TPM4	$3{\cdot}0 \times 10$	0	1·5	0
3	PrM4	$2{\cdot}0 \times 10^4$ s	1×10^3 s	1×10^4 s	5×10^2 s
4	TPM4+PrM4	$6{\cdot}0 \times 10^5$	4×10^4	3×10^5	2×10^4
5	TP36	$1{\cdot}0 \times 10^2$	0	5	0
6	Pr36	$3{\cdot}2 \times 10$	0	1·2	0
7	TP36+Pr36	$3{\cdot}6 \times 10$	0	4·6	0

Fraction TPM4 = DNA-containing fraction } From second-step proflavine-resistant
Fraction PrM4 = protein fraction } mutant
Fraction TP36 = DNA-containing fraction } From proflavine-sensitive *E. coli*
Fraction Pr36 = protein fraction } strain 36
s = small colonies

The experiment was carried out five times, each time with new preparations from *Escherichia coli* M4. On three occasions preparations from the resistant organisms gave positive results as described, on the other two occasions no increase in the number of resistant organisms was observed. We also carried out two similar experiments with preparations made from *E. coli* M1, a first-stage mutant isolated by replica plating. One of these gave, in 10^7 viable organisms, 10,000 organisms resistant to 50 μg. proflavine/ml. A second experiment gave no increase in resistant organisms. In each experiment, controls containing preparations from the sensitive strain 36 were also used. In none of these was there an increase in the number of resistant organisms, nor did the protein fraction from the sensitive strain decrease the viable count. The resistant organisms produced in the presence of the protein fraction alone always gave small colonies; we do not know why.

Substitution of the protein fraction. The activity of the protein fraction alone may be explained by its content of TP; the protein fraction showed a weakly positive Stumpf reaction. According to Mayers & Spizizen (1954) about 30% of the DNA comes down with the protein fraction. Since, however, the TP was itself not active, it seems as if some other fraction necessary for transformation was present in the protein fraction. This might be due to some unspecific action of the protein. It might also be due to the Duponol, since some of this was precipitated with the acetate and was subsequently found in the protein fraction. We attempted to substitute a variety of other substances for the protein fraction, to see whether they would also activate the TP. We used rabbit serum (as an unspecific protein), Duponol+acetate mixture, and deoxycholate (as an alternative surface-active agent). They were used in the following quantities: rabbit serum, 0·1 ml. of a 1/20 dilution; Duponol-acetate, 0·1 ml. of a mixture of 1 ml. Duponol 6% (w/v) and 5 ml. 3M-sodium acetate; deoxycholate, 0·1 ml. 0·1% (w/v) solution. Duplicate cultures were set up in the usual way. Each contained 5 ml. fluid, with or without TP from the sensitive or resistant (strain M 4) organisms, and with or without the protein fraction or the substitute for it. They were inoculated with the sensitive strain 36, incubated for 18 hr., and counts on pooled duplicate samples made of total viable organisms and of resistant organisms on plates with proflavine 20 and 50 μg./ml. Cultures which showed increased resistance were subcultured overnight in nutrient broth without proflavine. These were then tested on gradient plates with 100 μg. proflavine/ml. and for cross-resistance to chloramphenicol on gradient plates containing 15 μg. chloramphenicol/ml.

Table 2. *Transformation of sensitive* Escherichia coli 36 *with extracts of resistant (mutant) organisms*

			Proflavine (μg./ml.)		Proflavine (μg./ml.)	
			20	50	20	50
Culture	DNA* fraction	Protein fraction or substitute	Organisms/ml. resistant to proflavine		Organisms /10^{7} total viable resistant to proflavine	
1	—	—	$2{\cdot}0 \times 10^{2}$	0	$1{\cdot}0 \times 10$	0
2	—	Duponol	$2{\cdot}0 \times 10^{2}$	0	$1{\cdot}0 \times 10$	0
3	—	Serum	$1{\cdot}0 \times 10^{2}$	0	5	0
4	—	Deoxycholate	$1{\cdot}3 \times 10^{2}$	0	6	0
5	—	PrM4	$5{\cdot}0 \times 10^{4}$ s	1×10^{3} s	$2{\cdot}5 \times 10^{4}$ s	5×10^{2} s
6	—	Pr36	$4{\cdot}0 \times 10^{2}$	0	$2{\cdot}0 \times 10$	0
7	TPM4	—	$1{\cdot}1 \times 10^{2}$	0	5	0
8	TPM4	Duponol	$5{\cdot}2 \times 10^{5}$	4×10^{4}	$2{\cdot}6 \times 10^{5}$	2×10^{4}
9	TPM4	Serum	$8{\cdot}0 \times 10$	0	4	0
10	TPM4	Deoxycholate	$1{\cdot}0 \times 10^{2}$	0	5	0
11	TPM4	PrM4	$6{\cdot}0 \times 10^{5}$	1×10^{4}	$3{\cdot}0 \times 10^{5}$	5×10^{4}
12	TP36	—	$1{\cdot}2 \times 10^{2}$	0	6	0
13	TP36	Duponol	$3{\cdot}0 \times 10^{2}$	0	$1{\cdot}5 \times 10$	0
14	TP36	Serum	$2{\cdot}4 \times 10^{2}$	0	$1{\cdot}2 \times 10$	0
15	TP36	Deoxycholate	$1{\cdot}5 \times 10^{2}$	0	$7{\cdot}5$	0
16	TP36	Pr36	$4{\cdot}5 \times 10^{2}$	0	$2{\cdot}3 \times 10$	0

s = small colonies.
* See Table 1.

Table 2 shows that no increase in resistant organisms occurred with TP or with protein fraction, or with both together, from the sensitive organisms (cultures 6 and 12–16). As in previous experiments, the TP from the resistant organisms was also not active by itself (culture 7), but the protein fraction was active (culture 5). Still greater activity was shown by a mixture of TP and the protein fraction from the resistant organisms (culture 11). In this experiment, too, there was a decrease of the viable count by about 90 % in cultures containing the protein fraction (cultures 5 and 11). Neither serum nor deoxycholate replaced the protein fraction (cultures 9 and 10). On the other hand, Duponol replaced the protein fraction and caused a similar increase in the number of resistant organisms (culture 8). Duponol alone was not active. We do not know why there was a decreased viable count only in those cultures which contained protein fraction from resistant organisms or Duponol.

This experiment was carried out eleven times. In four, with three different preparations, results were obtained similar to those described in Table 2. The other seven experiments, with four different preparations, gave no increase in the number of resistant organisms.

Specificity of TP. The only instances where sensitive organisms were made to give resistant organisms were those in which TP was prepared from resistant organisms. Two strains of these were used; both were isolated by replica plating and had never been in contact with proflavine. In no instance were extracts of sensitive organisms active. In four of the experiments described above, additional culture tubes were included in which TP was replaced by commercial DNA. It was supplied at 500 μg./ml., and we purified it in the same way as we prepared TP from *Escherichia coli*. When acid ethanol was added, a granular precipitate was formed instead of the stringy precipitate formed from bacterial extracts. In two of the four experiments, transformation occurred with the TP from resistant organisms, but not with commercial DNA + Duponol. In the other two experiments no transformation occurred even with TP from resistant organisms.

Stability and cross-resistance of transformed organisms. In successful experiments cultures containing resistant organisms were subcultured three times in broth. They still gave a considerable increase in the number of colonies growing on proflavine plates when tested after this time. After one transfer in broth the cultures were tested on gradient plates containing 100 μg. proflavine/ml. They gave streaks about half way across the plate as compared with streaks right across the plates which were given by the donor organisms of strain M4 which were used in transformation. The streaks were similar to those given by the first-step mutant M1 when diluted 1/100 with sensitive organisms. They also gave streaks on plates containing 15 μg. chloramphenicol/ml., indicating a resistance to this drug about 60 % of that of strain M4.

Synchronized cultures. Hotchkiss (1951) showed that transformation of pneumococci occurred only during a short period of the division cycle. We therefore attempted transformation to proflavine resistance in synchronized cultures of sensitive *Escherichia coli* strain 36, by extracts of resistant strain M4. Four experiments were carried out, in each of which synchronization was attempted by cooling (see previous paper). In two of these, there was an increase in the number of resistant organisms at one interval after the cooling, in the presence of TP from resistant organisms + Duponol. In the other cultures, at different intervals or without Duponol + TP from resistant organisms, there was no such increase. These results suggest that transformation may occur at particular times of the division cycle, but the lack of reproducibility of transformation, which is a feature of all this work, makes it impossible at present to be definite about this.

Inactivation of TP by DNAase. In the four synchronization experiments, tubes were included in which the TP had been treated with a commercial preparation of DNAase, purified according to the method of McCarty & Avery (1946). In none of these experiments, including the two in which transformation appeared, was there an increase in number of resistant organisms in the tubes containing the treated TP.

Role of Duponol. TP from resistant organisms was only active when Duponol was present. We considered the following ways in which Duponol might act.

(*a*) Selective action of Duponol. McCarty & Avery (1946) postulated that the demonstration of transformation requires the presence of an agent which will favour the growth of the transformed organisms in the presence of those which have not been transformed. In our experiments we found that Duponol alone did not increase the number of proflavine-resistant organisms, nor did it do so in presence of TP from sensitive organisms, commercial DNA or TP from resistant organisms treated with DNAase. If Duponol acts as a selective agent, we should have to assume that it does so only on newly transformed organisms. We showed by gradient plates that proflavine-resistant organisms were cross-resistant to Duponol; but the converse is not true. With the Duponol-sensitive strain, 0·5 ml. Duponol + acetate added to 10 ml. agar decreased the length of streak by about one-third. There was no effect with 0·1 ml., and a decrease by two-thirds of the length with 1·0 ml. The nine Duponol-resistant strains tested, and the three proflavine-resistant strains, all gave streaks right across the plates with the highest Duponol concentration. The amount of Duponol + acetate in the transformation experiments was equivalent to 0·2 ml./10 ml. medium.

(*b*) Action of Duponol on the bacterial surface. Austrian (1952) pointed out that there is a correlation between the transformability of some strains of Pneumococcus and their high solubility in bile salts. We might then envisage that the low concentration of Duponol, which in higher concentrations is lytic, might increase the permeability of the organisms to the transforming principle. We found, however, that organisms sensitized by Duponol were not transformable, and the deoxycholate did not act like Duponol. Further experiments, however, would be needed to rule out this possibility altogether.

(*c*) Inhibition of DNAase by Duponol. If the recipient organisms produce sufficient DNAase there might be a destruction of the transforming principle before it had time to act. We tested this possibility. Cultures of *Escherichia coli* strain 36 were grown in pour plates with and without 0·2 ml. Duponol + acetate mixture. Before incubation, just before the agar had set, some of the fibrous TP from *E. coli* strain M4 was placed in the agar. There was significantly less digestion of the TP in the plate containing Duponol. These results support the view that the mechanism by which Duponol activates the transforming principle is by inhibiting the digestion of the DNA therein by DNAase from the recipient organisms.

DISCUSSION

We summarize here the experiments which we carried out in attempting to demonstrate transformation to proflavine resistance by DNA preparations made according to the method of Mayers & Spizizen. Of the ordinary cultures, we have had a total of 221 in which transformation would not be expected. In none of these was there an increase in the number of resistant organisms. We had a total of 45 cultures in which transformation might have been expected. In 19 of these cultures there was a considerable increase in the number of resistant organisms. Of the synchronized cultures, we had a total of 63 in which transformation would not be expected, and was not found. We had 13 cultures in which, apart from the time from synchronization, the appropriate materials were present which might have given transformation. Two of these 13 cultures showed a considerable increase in the numbers of resistant organisms. Since we expected to find transformation only at certain times after synchronization, we could not expect as many positive results here as in the experiments in which we used non-synchronized cultures.

We have tested the resistance of the sensitive *Escherichia coli* strain 36 on a large number of occasions over 4 years. In no instance did an untreated culture show the presence of resistant organisms in anything like the number which we have reported as occurring in our successful transformation experiments.

We have clearly not yet determined the factors necessary for successful transformation. In two experiments, preparations which caused transformation did not do so after being stored 3 or 4 days in the refrigerator. On the other hand, a preparation which did not cause transformation when freshly prepared did so after 2 days in the refrigerator.

One factor may be the concentration of Duponol. In none of the four preparations in which 1% (w/v) Duponol was used, as in the experiments of Mayers & Spizizen (1954), was transformation achieved. In all the experiments which were successful, 6% (w/v) Duponol had been used.

All workers have stressed that not all strains or all individual organisms are capable of undergoing transformation. Our results may contribute something toward the understanding of some of the factors involved. If a culture is taken to consist of several substrains, of which only a small proportion is competent, then we feel that our technique of 'double replica plating' helps to isolate them. It offers a far less laborious method than the testing of hundreds of individual strains and we hope that other workers will be encouraged to try this method. In addition to competence of such strains, competence may depend on the period of the division cycle in which transformation is attempted. Our own few experiments suggest that this may be true for *Escherichia coli*, as Hotchkiss (1954) had found for Pneumococcus.

The evidence we offer that proflavine sensitivity may be transformed to proflavine resistance may be summarized as follows. (*a*) A large number of organisms from sensitive cultures have been made resistant after being brought into contact with preparations containing DNA from resistant organisms. One such preparation was made from a strain 'trained' to proflavine resistance; the other two preparations were from independently isolated mutants which had never been in contact with proflavine. (*b*) Commercial DNA, and preparations containing DNA from sensitive organisms, did not produce this effect. (*c*) Active extracts from resistant strains were no longer active after treatment with DNAase. (*d*) The change to resistance in the recipient organisms is heritable. In view of the difficulty we have experienced in reproducing our results, some of these findings need to be confirmed. We believe, nevertheless, that we have demonstrated true transformation to proflavine resistance and that this shows the genetic nature of the resistance in the organisms used as donors. These were both a 'trained' strain, and two strains isolated by replica plating. Our studies give support to the suggestion of a multistep pattern of proflavine resistance. A second-step mutant, derived without contact with drug, transformed to a lower resistance of about that of the first-step mutant. This recalls the similar observation of Hotchkiss (1951) with transformation to penicillin resistance in Pneumococcus. Our experiments support the use we have made of studies in cross-resistance as evidence of genetic change. Organisms transformed to proflavine resistance were cross-resistant to chloramphenicol in the same way as proflavine-resistant organisms which originated by mutation. Since it is generally held that transformation occurs of only one character at a time, this observation implies that the same genetic determinant can be responsible for resistance to both drugs.

Whilst we have worked with only one organism + drug system, it seems reasonable to imagine that the phenomena we have studied are not unique. Let us then consider the ways in which a sensitive culture of bacteria may develop resistance when challenged with a toxic agent such as a drug. We believe we have demonstrated five factors which may determine whether bacteria are able to grow in the presence of a drug: (1) alteration in toxicity of the drug due to environmental changes such as in pH; (2) the stage of development of the culture, which we have called the cultural phase; (3) a small increase in resistance of the organisms by adaptation; (4) spontaneous development of resistant mutants; (5) transformation of sensitive organisms to resistance by extracts of resistant organisms. We also have evidence, though not so strong, of clonal variation and of adaptation (induction) to high degrees of resistance. Excluding these, however, we can begin to see that there are many interrelated factors, occurring together or in sequence, which will determine whether a bacterial culture can grow in the presence of a drug. First, there are the factors related to the environment rather than to the bacteria. A given concentration of drug will prevent growth in particular stages of cultural growth, but not in others. In our experiments, we showed that one concentration of proflavine is inhibitory in the late logarithmic phase but not in the lag phase. Again, an inhibitory concentration of drug may be made non-inhibitory simply by increasing the acidity of the culture. In appropriate conditions, then, even sensitive bacteria may be able to survive and perhaps divide, giving an increasing chance of the organisms developing resistance.

This brings us to the second series of factors, related to the bacteria themselves—adaptation, mutation and transformation. If there are enough of the bacteria present, there is a chance that some spontaneously-occurring resistant mutants are also present. These might then grow in the presence of drug so as to give a new and resistant population. It is possible, however, that none, or only a few, are originally present. In these conditions, the phenomenon of adaptation may be of importance. Some of the bacteria, perhaps in particular stages of their division cycle, may be able to divide in small or moderate concentrations of the drug. Though, as we have seen, such resistance may be temporary, in the sense that it is lost when the drug is removed, it persists so long as the drug is present. The longer the bacteria go on dividing, the greater the chance that spontaneous mutation to resistance will occur.

The existence of the phenomenon of transformation gives a bacterial population an additional chance of survival. For bacteria are constantly dying and being lysed by a variety of conditions not necessarily related to the presence of the toxic agent. If then a population happens to contain a moderate number of genotypically-resistant organisms, there is some chance that their death does not involve the complete loss of the property of resistance. The transfer of this property to sensitive organisms means that the population as a whole continues to contain some individual cells which can withstand the drug.

We are clearly not yet able to describe in any precise way the events which may lead to the preservation of a population of bacteria which is challenged by a drug. More especially, it would at this stage be premature to extend these considerations to natural situations, where different micro-environments may exist, and where other species of bacteria and their products would undoubtedly influence the interaction between bacteria and toxic agent. Nevertheless, the multiple factors determining resistance which we know to exist make it possible to begin to visualize these events in a way which is not possible in terms of the concept of a single factor-determining resistance, which has often been suggested for at least single bacteria + drug systems.

This paper completes the series. A preliminary account was given at a Ciba Foundation Symposium ('Drug Resistance in Micro-organisms', Churchill, London, 1957).

REFERENCES

Alexander, H. E. & Leidy, G. (1950). Transformation type specificity of *H. influenzae*. *Proc. Soc. exp. Biol., N.Y.*, **73**, 485.

Alexander, H. E. & Leidy, G. (1951). Determination of inherited traits of *H. influenzae* by desoxyribonucleic acid fractions isolated from type-specific cells. *J. exp. Med.* **93**, 345.

Alexander, H. E. & Leidy, G. (1953). Induction of streptomycin resistance in sensitive *Haemophilus influenzae* by extracts containing desoxyribonucleic acid from resistant *Haemophilus influenzae*. *J. exp. Med.* **97**, 17.

Alexander, H. E. & Redman, W. (1953). Transformation of type specificity of Meningococci. *J. exp. Med.* **97**, 797.

Austrian, R. (1952). Bacterial transformation reactions. *Bact. Rev.* **16**, 31.

Boivin, A. (1947). Directed mutation in *Colon bacilli* by an inducing principle of desoxyribonucleic nature: its meaning for the general biochemistry of heredity. *Cold Spr. Harb. Symp. quant. Biol.* **12**, 7.

Ephrussi-Taylor, H. (1951). Transformations allogenes du pneumocoque. *Exp. Cell. Res.* **2**, 589.

Griffith, F. (1928). The significance of pneumococcal types. *J. Hyg., Camb.* **27**, 113.

Hotchkiss, R. D. (1951). Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. *Cold. Spr. Harb. Symp. quant. Biol.* **16**, 457.

Hotchkiss, R. D. (1954). Cyclical behaviour in pneumococcal growth and transformability occasioned by environmental changes. *Proc. nat. Acad. Sci., Wash.* **40**, 49.

McCarty, M. & Avery, O. T. (1946). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. exp. Med.* **83**, 89, 97.

Mayers, V. L. & Spizizen, J. (1954). The isolation of desoxyribonucleic acid from bacteriophages by an improved method. *J. biol. Chem.* **210**, 877.

Stumpf, P. K. (1947). A colorimetric method for the determination of desoxyribonucleic acid. *J. biol. Chem.* **169**, 367.

Thornley, M. & Yudkin, J. (1959). The origin of bacterial resistance to proflavine. 2. Spontaneous mutation to proflavine resistance in *Escherichia coli*. *J. gen. Microbiol.* **20**, 365.

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The Serology and Pathogenicity of the Genus *Chromobacterium*

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SUMMARY: Mesophilic strains of the genus *Chromobacterium* were found to cross-agglutinate extensively, and much of this appears to be due to common rough somatic antigens. The psychrophilic strains also cross-agglutinated to some extent. There was little cross-agglutination between mesophils and psychrophils. Neither mesophils nor psychrophils showed clear-cut antigenic subgroups. Many mesophilic strains, whether isolated from naturally-occurring cases of infection or from water, were found to be virulent for experimental animals. The most virulent were strains from two cases of human infection, which had an LD50 dose for guinea-pigs of *c.* 5×10^6 viable organisms. The virulent strains did not form a homogeneous antigenic group. Cultures of mesophils contained an endotoxin, but no exotoxin was found. The experimental disease may be an acute, rapidly fatal septicaemia, or a more chronic disease with multiple abscess formation like that found in natural infections; occasionally a local abscess with subsequent recovery was the only result of the injection of cultures.

The generic name *Chromobacterium* is used here in the restricted sense of the 'Bacterium violaceum group', excluding red and yellow chromogens such as 'Bacillus prodigiosus' (*Serratia marcescens*). There are two main types of Chromobacterium, both of which in the past have usually been called *Chromobacterium violaceum*, and which may conveniently be regarded as two species; but since the correct names for these two species are uncertain (Sneath, 1956*a*), they are referred to here as the 'mesophils' and the 'psychrophils'. The former usually ferment glucose anaerobically, are markedly proteolytic, facultatively anaerobic, and give a positive egg-yolk reaction, but do not hydrolyse aesculin. The psychrophilic organisms produce acid from glucose but only aerobically, are poorly proteolytic, strictly aerobic and hydrolyse aesculin but do not produce an egg-yolk reaction. Infections by Chromobacterium strains are rare, and are produced only by mesophilic strains. Most of the recorded cases to date were listed by Sneath, Whelan, Singh & Edwards (1953). Other instances are those described by Floch & de Lajudie (1943) in cattle, and by Audebaud, Gazin, Ceccaldi & Merveille (1954) in a monkey; the strains examined by these authors have unfortunately been lost (Floch, personal communication; Audebaud, personal communication). In addition, some strains were kindly sent to us which had been isolated from cases of disease in cattle and pigs by Sippel, Medina & Atwood (1954), in a pig by Sippel (1955) and in man by Darrasse, Mazaud, Giudicelli & Camain (1955). Further records of infection were briefly reported by Broudin (1922) in a wild boar, by Joubert & Nguyen-van-Liem (1957) in buffaloes: Dr J. P. F. Whelan (personal communication) recently isolated a strain in pure culture from the brain of a pet monkey which was thought to have died of rabies in Singapore but from which no rabies virus was recovered, and which was presumably a case of chromobacterial septicaemia.

Most authors who have described relevant infections have made some study of the pathogenicity of their strains and have examined their virulence for laboratory animals by the injection of relatively large inocula (e.g. Woolley, 1904, 1905; Schattenberg & Harris, 1942), but no estimates of virulence based on viable counts have, as far as we can ascertain, been published. There is little work on the serology of the group other than that of Bampton (1913).

METHODS

Strains and media. The strains used were those described by Sneath (1956*a*) and the media were prepared as described in that paper. The nutrient broth used was that of Hartley (1922) and nutrient agar was made by adding to this 1·5 % (w/v) of Japanese agar. The strains BHR/1 and BNR/1 were rough variants of the strains BH and BN respectively. The rough variants were obtained by growth of the latter strains in nutrient broth containing, respectively, sterile anti-BH or anti-BN ‘O’ sera. They were only slightly rough (both colonially and antigenically) as compared with very rough strains such as SH or AM.

Serological methods. For the preparation of suspensions for immunization and agglutination, mesophilic strains were grown routinely for 24 hr. at 30° and psychrophilic strains for 48 hr. at 25°. Antisera were prepared by injecting killed organisms intravenously into rabbits three times a week until the animals had received six injections, in doses increasing from *c.* 10^7 to *c.* 5×10^8 organisms; the animals were bled 10 days after the last injection. The sera were either stored at $-20°$, or, after mixing with an equal volume of glycerol, at 4°. No decrease in titre has been noticed after five years of storage by either method.

‘O’ antisera were prepared by injecting suspensions washed from 24 hr. nutrient agar slope cultures and heated at 100° for 2 hr. ‘OH’ antisera were prepared by injecting nutrient broth cultures killed by the addition of sufficient formalin to give a final concentration of 0·04 % (w/v) of formaldehyde.

Agglutinable suspensions were diluted with 0·17 (w/v) sodium chloride solution (this was used to decrease the autoagglutination of rough strains) until they had an opacity 0·55 times that of the International Opacity Standard (World Health Organization, 1954), i.e. 5·5 Opacity Units, which is about equivalent to an optical density of 0·33 in a 1 cm. cell with an Ilford H508 neutral grey filter. Titrations were performed by diluting antisera in twofold steps in 0·17 % (w/v) NaCl, and mixing 0·25 ml. volumes of the dilutions with 0·25 ml. volumes of bacterial suspension in Dreyer's tubes, and then incubating them. The titre recorded is the reciprocal of the final dilution of antiserum in the last tube which showed agglutination visible to the naked eye.

To demonstrate O agglutination anti-O serum was mixed with living suspensions of organisms grown in nutrient broth, and incubated for 4 hr. at 56° followed by 18 hr. at room temperature. To demonstrate H agglutination due to polar flagella, 'OH' antiserum was incubated at 56° for 18 hr. with suspensions of organisms grown in nutrient broth and formolized as described above. To demonstrate H agglutination due to lateral flagella, the 'H' or 'OH' sera were incubated for 2 hr. at 56° with formolized suspensions of bacteria grown on nutrient agar plates for 18 hr. The suspensions for H agglutination were examined by flagella staining to confirm the flagellation.

During the latter part of this work a large number of psychrophilic strains were received and it was not practicable to make antisera to them all. Antisera were made only to a few strains and the numerous combinations of antiserum and strain were first tested by slide agglutination with concentrated serum and living suspensions from nutrient agar plates. Only those mixtures which showed agglutination on the slides were then titrated in tubes. Such slide tests revealed agglutination due to polar H antigens as well as the other antigens; it is unlikely that any significant cross-reactions were missed by this screening procedure.

Pathogenicity. Rabbits (2 kg.), guinea-pigs (250 g.) and white mice (20 g.; Porton strain) were used. The inocula were aerobic nutrient broth cultures grown for 24 hr. at 37° (for the psychrophilic strain NT at 25° for 48 hr.). Tenfold dilutions were made in 0·03 M-potassium phosphate buffer (pH 7·6) and 0·1 ml. volumes of suitable dilutions were inoculated into animals within 30 min. of making the dilutions. The animals were observed for 1 week, when the death-rate was scored. Viable counts of the inocula were made by the drop method (Miles & Misra, 1938) on nutrient agar plates containing 5% (v/v) peptic digest of sheep's blood (Fildes, 1920). The diluent was 0·03 M-potassium phosphate buffer (pH 7·6). Colonies were counted after incubation for 24 hr. at 30° (48 hr. at 20° for psychrophilic strains).

Autopsy and histology. Animals which died from infection or toxic effects were autopsied. Samples of blood from the heart and tissue samples from any visible lesions in the liver and spleen were inoculated into nutrient broth. After incubation at 30° for 48 hr. (20° for 48 hr. for psychrophilic strains) the broth cultures were plated out on blood agar plates to confirm the presence of the organism. Material for histology was fixed in 10% (v/v) formol saline; paraffin sections were stained by routine methods.

RESULTS

Antigenic structure

O antigens. The treatment of the suspensions used for agglutination had a considerable effect on the pattern of agglutination with different sera. Both ethanol treatment and heating of organisms at 100° usually caused a decrease in titre of O antiserum against the homologous strain, while cross-reactions with sera against other strains became more marked. Some of the 'rough' strains became more strongly autoagglutinable after heating to 100°. For-

Table 1. *Titration of O agglutination of mesophilic strains of Chromobacterium with antisera prepared against the corresponding organisms*

'Q' antisera titrated against living broth cultures incubated for 4 hr. at 56° and then for 18 hr. at room temperature, the figures give the reciprocals of the dilutions of the antisera which gave agglutination.

Suspensions of strains	Antisera against strains															
	SL	BN	BH	BNR/1	BHR/1	LG	FH	RT	MH	AM	SH	LW	MW	TV	DK	MK
	Reciprocal of antiserum dilution															
SL (S)	**640**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BN (S)	80	**160**	320	20	40	0	0	0	20	0	0	0	40	160	160	320
BH (S)	0	160	**320**	20	0	0	0	0	20	0	0	0	0	0	0	0
BNR/1 (r)	—	160	160	**640**	320	320	320	0	—	0	0	320	320	320	—	320
BHR/1 (r)	—	0	0	0	**80**	320	320	0	—	0	0	0	0	0	—	0
LG (S)	0	0	0	0	40	**1280**	160	0	80	0	0	0	0	0	0	0
FH (S)	0	320	20	160	320	320	**5000**	320	160	0	0	0	0	0	0	0
RT (R)	0	0	80	0	40	320	80	**320**	320	0	0	0	0	160	0	80
MH (S)	0	0	0	0	0	0	0	40	**640**	160	80	80	40	20	0	10
AM (R)	0	0	40	40	80	0	160	0	80	**1280**	0	80	0	160	160	320
SH (R)	0	0	0	0	0	160	320	0	160	0	**160**	0	0	0	20	0
LW (R)	0	0	20	40	20	0	320	320	320	0	640	**640**	0	80	0	80
MW (R)	0	0	0	0	0	0	20	0	160	160	40	0	**640**	320	20	40
TV (S)	0	0	40	0	0	0	0	0	0	0	0	0	160	**640**	0	40
DK (S)	0	0	80	0	40	0	0	0	0	0	0	0	0	0	320	80
MK (S)	0	0	0	0	0	0	0	0	0	0	0	320	0	0	160	**1280**
RV (r)	0	20	0	0	0	20	0	20	0	40	0	20	10	40	160	40
TA (S)	0	0	0	0	0	0	0	0	0	0	0	0	40	40	0	20
TB (S)	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0
PT (S)	0	10	20	20	0	0	0	0	0	0	0	0	0	0	0	40

0 = less than 20. — = not tested.

The approximate degree of 'Roughness' of the strains is indicated by the symbol in parentheses after the strain letters: (R) = markedly rough, giving considerable autoagglutination in 0·9 % NaCl; (r) = slightly rough, giving only slight autoagglutination; (S) = smooth, giving no autoagglutination.

The strains from cases of natural infections and their source are: SL, Sealy strain from pig (Sippel *et al.* 1954); BH and BN from man (cases 11 and 12 in Sneath *et al.* 1953); SH, Shahan strain from man (Black & Shahan, 1938); DK, from man (Darrasse *et al.* 1955); RV, Reeves strain from cattle (Sippel *et al.* 1954) and PT, Porter strain from pig (Sippel, 1955).

molized polar flagellated suspensions gave titres similar to those obtained with living suspensions, but partial inhibition of O agglutination was found when formolized peritrichous suspensions were used. The results reported here were obtained with living suspensions; they are recorded in Tables 1 and 2 which show, respectively, the agglutination titres of antisera against mesophilic strains with suspensions of mesophilic strains, and the titres of antisera against psychrophilic strains with suspensions of psychrophilic strains.

Table 2. *The titration of O antisera prepared against psychrophils with psychrophilic strains of Chromobacterium*

The 'O' antisera were titrated against living broth cultures incubated for 4 hr. at 56° and then for 18 hr. at room temperature.

All these psychrophilic strains were culturally 'smooth'.

	Antisera against strains						
	NT	EC	NC	PB	HB	HE	MB
Suspensions of strains	Reciprocal of dilution of antisera						
NT	**160**	0	0	0	0	0	0
EC	0	**160**	40	0	0	0	0
NC	0	0	**320**	160	0	0	0
PB	0	0	10	**160**	0	0	0
HB	0	0	0	0	**2560**	0	0
HE	0	0	0	0	40	**80**	0
MB	0	0	0	0	0	0	**160**
EA	0	80	0	0	0	0	0
HD	0	0	160	80	0	0	0
GA	0	0	80	0	0	0	0
MA	0	0	160	0	0	0	0
DA	0	0	0	40	0	0	0
IN	0	0	0	20	10	0	0
HA	0	0	0	0	40	10	0
EB	0	0	0	0	0	0	0
MC	0	0	0	0	0	0	0
HC	0	0	0	0	0	0	0
HF	0	0	0	0	0	0	0
CA	0	0	0	0	0	0	0
RU	0	0	0	0	0	0	0

0 = less than 10.

There were a few instances of definite cross-agglutination between mesophilic and psychrophilic strains, and the tests are therefore not given in table form; these instances of cross-agglutination are listed below. Probably most of the weak cross-reactions were not due to common antigens, since normal rabbit sera often agglutinated some strains of Chromobacterium to a titre of 20 or 40, but not higher.

(1) Agglutination of mesophilic strains by antisera against psychrophils: anti-NT had a titre of 160 with strain AM and of 40 with strain LW; anti-HE had a titre of 40 with strain AM and of 80 with strain MH; anti-EC had a titre of 20 with strains MH and DK; anti-MB had a titre of 80 with strain MH; anti-HB had a titre of 160 with strain MH.

(2) Agglutination of psychrophilic strains by antisera against mesophils: anti-LG had a titre of 320 with strain NT; anti-MK had a titre of 20 with strain NT.

It will be seen from Table 1 that the mesophilic strains did not fall into clearly marked groups, although there were several which were closely related, such as strains BH and BN, FH and LG, MW and TV. It is also seen that rough strains cross-agglutinated more often than did smooth strains. It seems probable that this was due to the uncovering of deep somatic antigens possessed in common, since the rough variants of strains BH and BN (BHR/1 and BNR/1 respectively) agglutinated with sera which did not agglutinate their parent strains. Leifson (1956) separated the mesophilic strains which do not produce acid from carbohydrates under anaerobic conditions as a distinct species. Strains of this biochemical pattern are RT, MH, SH and LW; serologically they do not seem to form a distinct group. It will be seen from Table 2 that many of the antisera to psychrophils were almost strain specific, but that there were sufficient cross-reactions to confirm that the psychrophilic strains formed an antigenically related group. Bearing in mind the limited number of antisera and the absence of rough strains, the general behaviour of the psychrophils appears to be much the same as was found with the mesophils.

H antigens. The results of agglutination of broth cultures by 'OH' antisera (at 56° for 4 hr.) were usually very similar to those obtained with 'O' antisera. This observation was explained when it was found that the broth cultures usually possessed only polar flagella, which do not give good agglutination unless the tests are incubated at 56° overnight. The agglutination observed after 4 hr. was therefore mainly O agglutination. The preparation of pure polar 'H' antisera has not been possible, but it was shown by Sneath (1956*b*) that the antigens of the lateral flagella ('peritrichate' flagella) are different from the polar flagellar antigens. The typical H agglutination found with peritrichate organisms can be obtained using 'OH' antisera and peritrichate suspensions (consisting of organisms which possess both a polar flagellum and also lateral flagella) of young cultures on agar. The only detailed work with H antigens was with five of the strains from natural infections (sources noted in Table 1). It was of interest to see whether these strains possessed common H antigens. The 'OH' antisera, although prepared by immunizing with polarly flagellate suspensions, contained enough lateral H antibody to enable them to be used (after removing O and polar H antibodies by absorption with homologous polarly flagellate organisms, Sneath (1956*b*)). The existence of two forms of flagella also explains why some slide tests (with nutrient agar cultures, which would yield peritrichously flagellate organisms) were positive when tube tests (with polarly flagellate organisms from broth cultures) were negative.

A point of interest is whether there is any antigen common to the more virulent strains or to those which caused natural infections. The strains isolated from natural infections are strains BH, BN, SH, SL, DK, RV and PT (see Table 1), while the most virulent of those isolated from other sources are strains LG and MK (see Table 4). There was no evidence of an O antigen (see

Table 1) common to these strains. Some of these strains were also examined for polar and lateral flagellar agglutination. The results are given in Table 3, in which it is seen that there appeared to be no H antigen common to all the strains examined although strains BN and SL had a lateral H antigen in common.

Table 3. *Agglutination of Chromobacterium strains due to flagellar antigens*

I. Agglutination due to O and polar H antigens. Agglutination titres of antisera containing O and H antibodies with living suspensions possessing polar flagella but no lateral (peritrichous) flagella, incubated at 56° for 18 hr.

	Antisera against strains				
Suspensions of strains	BH	BN	SL	SH	DK
	Reciprocal of dilution of antisera				
BH	**80**	160	160	80	160
BN	1280	**320**	640	160	40
SL	10	80	**640**	0	0
SH	80	40	20	**640**	10
DK	40	160	80	40	**640**

II. Agglutination due to lateral (peritrichous) H antigens. Agglutination titres of H antisera (absorbed with homologous boiled organisms to remove O antibodies) with suspensions possessing lateral (peritrichous) flagella, incubated at 56° for only 2 hr. to avoid agglutination due to polar flagella.

	Antisera against strains				
Suspensions of strains	BH	BN	SL	SH	DK
	Reciprocal of dilution of antisera				
BH	No peritrichous suspensions obtainable				
BN	1280	**160**	160	0	0
SL	640	40	**80**	10	0
SH	0	0	0	**320**	0
DK	0	0	0	0	**1280**

0 = less than 10.

Antigenic relationship to other chromogenic bacteria. All the antisera against Chromobacterium strains were slide-tested against living suspensions of two strains of Whitmore's bacillus, *Pseudomonas iodinum* (strains TE and RE), *Corynebacterium viscosum* (strain GR) and six stock strains of 'Bacillus prodigiosus' (*Serratia marcescens*). No agglutination was found in any instance. Polyvalent 'OH' antisera against six strains of 'Bacillus prodigiosus' and an antiserum to Whitmore's bacillus did not agglutinate any strain of Chromobacterium. Strain TI (*Chromobacterium ianthinum* of Gilman, 1953) was tested with all the Chromobacterium antisera, and an OH antiserum against TI was tested with all Chromobacterium strains: a few doubtful reactions were found which were negative when tested by tube agglutination. These negative results are in keeping with the opinion that none of these other chromogenic organisms belong to the genus *Chromobacterium* (Sneath, 1956*a*); nor does Whitmore's bacillus.

Phase variation. Strain DK was examined to discover whether it possessed alternative antigenic phases of the lateral flagella, analogous to the Salmonella flagellar antigen phases, but no evidence of this was found by using standard techniques.

Pathogenicity

The majority of the mesophils were tested for pathogenicity, and it may be seen from Table 4 that the virulence of different strains varied widely, from highly virulent strains such as BN and BH, to strains like AM and the psychrophil NT, which were only lethal in huge doses and which probably caused death by intoxication. None of the rough strains was very virulent, and two rough variants of virulent strains, BHR/1 and BNR/1, had a greatly decreased virulence as compared with the parental strains.

Post-mortem and histological appearances. With highly virulent strains the injection of a large dose of living organisms (*c.* 5 times the LD 50 dose) caused death by septicaemia, often in 18 hr. The only post-mortem appearances were congestion of the abdominal viscera, with a slightly enlarged spleen, some general subcutaneous congestion and a little turbid or blood-stained fluid in the pleural and peritoneal cavities. After the subcutaneous injection of living organisms, there was oedema, often gelatinous, and congestion around the inoculation site with perhaps some haemorrhage. The less virulent strains gave smaller local lesions. The organism was readily recovered from the blood and all the organs. Histological changes were very slight.

The effect of doses of living organisms near the LD 50 dose was variable. Some animals developed septicaemia, some showed only a necrotic local lesion (after subcutaneous injection) which might ulcerate, and then slowly heal. A few animals developed pyaemia as is seen in man and in swine, with fever, anorexia, dyspnoea and rapid wasting, usually causing death after some days. At autopsy, abscesses were found in the liver and commonly also in the lungs and spleen, from which the organism was readily cultivated. The abscesses were similar in histological appearance to those seen in natural infections. The predominant lesion was necrosis, with a cellular reaction composed of polymorphonuclear leucocytes with a moderate number of lymphocytes and a few macrophages and plasma cells. Bizarre and distorted nuclei were often seen at the abscess edge, and the lesions contained many Gram-negative bacilli, sometimes slightly curved, which showed slight bipolar staining. These findings are similar to those of earlier workers (e.g. Woolley, 1904, 1905; Sippel, 1955).

Increase of virulence on passage. Several strains were passed five times through guinea-pigs, using large doses (*c.* 10^9 organisms) grown from heart blood of the animals, and their virulence was tested again. The results are summarized in Table 4, which shows the approximate LD 50 doses before and after passage. In a few cases the virulence was significantly but not greatly raised.

The effect of mucin in enhancing virulence. Mucin is well known to increase the virulence of some bacteria on injection. This point was tested for us by Mr G. J. Harper, with strain BH. He found that the LD 50 dose for organisms in broth was about 2×10^6 viable organisms, while that for mucin broth was *c.* 8×10^2 organisms.

Table 4. *Virulence of Chromobacterium strains for mice and guinea-pigs*

The organisms were grown in nutrient broth cultures for 18 hr. at 37°, except for strain NT which was grown at 25° for 48 hr. Viable counts were made, and after making suitable dilutions each dilution was inoculated intraperitoneally into ten white mice or five guinea-pigs. The approximate LD 50 dose was obtained by interpolation. Some strains were passed five times in guinea-pigs and then re-tested.

	Test animal		
	Mice	Guinea-pigs	
		Before passage	After passage
	Approximate LD 50 dose of living organisms		
Strain			
FH	Over 10^8	10^9	10^8
MK	10^6	10^8	10^8
BH	2×10^6	10^7	10^6
BHR/1	10^7	5×10^9	5×10^8
BN	10^6	5×10^8	10^6
BNR/1	10^7	10^9	5×10^8
AM	10^8	2×10^{10}	—
MW	Over 10^8	5×10^8	—
TV	5×10^7	10^{10}	—
LG	10^7	10^9	10^7
LW	Over 10^7	2×10^{10}	—
SH	Over 10^7	10^9	10^8
RT	Over 10^7	5×10^9	10^8
NT	Over 10^7	2×10^{10}	—

— = not tested

Table 5. *The effect of mucin added to inoculum upon virulence*

Batches of ten mice were inoculated intraperitoneally with 0·5 ml. volumes of living suspensions of strain BH suspended in broth or in broth + 1·75% (w/v) mucin.

	Number of deaths within 7 days	
Number of viable organisms injected	Without mucin	With mucin
$8{\cdot}0\times10^6$	10	—
$8{\cdot}0\times10^5$	0	10
$8{\cdot}0\times10^4$	0	9
$8{\cdot}0\times10^3$	0	9
$8{\cdot}0\times10^2$	0	5
$8{\cdot}0\times10^1$	—	1
0	0	1

— = not tested.
Approximate LD 50 dose without mucin = 5×10^6 organisms.
Approximate LD 50 dose with mucin = 8×10^2 organisms.

Correlation of virulence with source of strain. The most virulent strains, BH and BN, had been isolated from cases of infection in man; the strains isolated from water in Malaya (FH, MK and LG), possess a not much smaller degree of virulence. Other workers (e.g. Gauducheau, 1907; Minett, 1911) also found strains from water to be virulent. Thus it appears that the more virulent water strains may cause natural infections in man.

Toxicity. That the lethal effects of the mesophils are not due entirely to the toxicity of the injected material is suggested by the fact that the LD 50 dose of living organisms of the more virulent strains is several hundred times lower than that of organisms killed by heat or formalin; the less virulent strains clearly cause death mainly by the toxicity of the inoculum. Dodd (1941) found that the toxic factor in cultures was increased by cultivations in an atmosphere of air containing 25% carbon dioxide; this toxic factor was filterable. We have not been able to confirm these observations with strain BN, with which we found no effect upon the LD 50 dose of viable organisms by cultivation in 25% (v/v) CO_2. Culture filtrates were non-toxic to mice in 1 ml. doses intraperitoneally; but strain BN was not the strain used by Dodd. We noted that heat-killed mesophil organisms appeared to be more toxic than formalin-killed organisms, since several rabbits were inadvertently killed by starting immunization to produce O antisera with too large a dose of heated organisms, while much larger doses of formolized organisms were well tolerated. Dodd also noted that killed organisms were more toxic when given intravenously than intraperitoneally or subcutaneously. It therefore seems possible that haemolysins (probably partly lecithinase, see Sneath, 1956*a*) are largely responsible. In any event the toxicity of the organism is not very great. With strain MK inoculated intraperitoneally in guinea-pigs, we found that 18 hr. broth cultures grown at 37° were not lethal when injected in 1 ml. volumes when the organisms had been killed by heat (100° for 30 min.) or removed by centrifugation or Seitz filtration, though broth cultures containing living organisms caused death in over half the animals at this dosage (equivalent to *c.* $9{\cdot}0 \times 10^6$ viable organisms). There was thus no evidence for the presence of an exotoxin, and only for a weak endotoxin.

It was shown (Sneath *et al.* 1953) that mesophilic strains of Chromobacterium produced considerable quantities of hydrogen cyanide; this may perhaps play a part in causing death in infections. But it was not possible to demonstrate by the method of Aldridge (1944) a rise in the concentration of (cyanide + thiocyanate) in the blood of rabbits which died of septicaemia.

DISCUSSION

The serological results show that Chromobacterium is similar to other Gram-negative bacteria in its antigenic properties. The O antigens of smooth strains tend to be more specific than the rough antigens: heat or ethanol tends to increase the auto-agglutinability of suspensions, and rough strains are of low virulence. The anomalous behaviour of the H antigens may be accounted for by the ability of most strains to develop lateral (peritrichous) flagella when grown on solid media, in addition to the single polar flagellum found on organisms grown in liquid media. The division of the genus into the mesophilic and psychrophilic groups (Leifson, 1956; Sneath, 1956*a*, 1957) is confirmed by the serology. It is notable that the pathogenic mesophils from cases of natural infection do not form a homogeneous antigenic group; they were previously shown not to form a homogeneous biochemical group (Sneath, 1956*a*). This is a disadvantage in epidemiological studies, such as that of Sippel (1955), where attempts were made to detect swine which had had subclinical infections, since the antibodies resulting from such infections would be mainly strain-specific and would probably not be detected by tests with suspensions of stock laboratory strains. There is some correspondence between the serology of the O antigens and the composition of the O polysaccharides from a number of these strain examined by Davies (1955), but the agreement is not as close as it is in Salmonella.

The disease produced by infection with mesophilic strains of Chromobacterium is similar in many ways to melioidosis, as was noted by Darrasse *et al.* (1955). The clinical picture is similar, and both conditions are generally pyaemias with multiple abscess formation, particularly in liver and lungs, and a high mortality rate. The causative organisms in both diseases appear to be found naturally in stagnant water (see Chambon, 1955), and are evidently of relatively low infectivity in nature. Epizootics with both organisms have been reported in swine (see Nguyen-Ba-Luong, 1956); the histological changes are also similar. However, Chromobacterium and Whitmore's bacillus appear to be quite distinct from each other, both culturally and antigenically.

Although apparently, rare Chromobacterium infections may be more common than is supposed, since the causative organism is likely to be dismissed as a contaminant. Sippel and colleagues studied an epidemic which involved over seventy pigs; they also found that antibodies to their strain was not uncommon in normal pigs. The disease may thus be of some veterinary importance. Sippel (1955) found that Aureomycin was useful for treatment, and Sneath *et al.* (1953) also had some evidence that this substance was efficacious in man. In the disease in pigs and cattle the evidence (reviewed by Sippel, 1955) suggests that the usual route of entry of the organism is by the mouth; but several authors have been unable to infect small laboratory animals by this route. Sippel *et al.* (1954) were able to infect pigs by mouth; pigs may be unusually susceptible to infection through the pharyngeal tissues, since the natural cases commonly show cervical adenitis. A similar behaviour is seen with anthrax infection in pigs. In man the site of entry appears to be sometimes through a wound (Schattenberg & Harris, 1942; Sneath *et al.* 1953), but more often it is unknown.

REFERENCES

ALDRIDGE, W. N. (1944). A new method for the estimation of micro quantities of cyanide and thiocyanate. *Analyst*, **69**, 262.

AUDEBAUD, G., GAZIN, M., CECCALDI, J. & MERVEILLE, P. (1954). Isolement d'un *Chromobacterium violaceum* à partir des lésions hépatiques observées chez un singe *Cercopithecus cephus*. Étude et pouvoir pathogène. *Ann. Inst. Pasteur*, **87**, 413.

BAMPTON, J. H. (1913). Über Violaceus und Membranaceus amethystinus. *Zbl. Bakt.* (I. *Abt. Orig.*), **71**, 129.

BLACK, M. E. & SHAHAN, J. (1938). *Bacillus violaceus* infection in a human being. *J. Amer. med. Ass.* **110**, 1270.

BROUDIN, L. (1922). Cited in 'Sur un microbe pathogène isolé des eaux de Saigon (*Bacillus violaceus acetonicus*)'. In *Les Instituts Pasteur d'Indochine*: *Centenaire de Louis Pasteur*, 1822–1895, pp. 196–8. Edited by P. N. Bernard. Saigon: Imprimerie Nouvelle Albert Portail.

CHAMBON, L. (1955). Isolement du bacille de Whitmore à partir du milieu exterieur. *Ann. Inst. Pasteur*, **89**, 229.

DARRASSE, H., MAZAUD, R., GIUDICELLI, P. & CAMAIN, R. (1955). Un cas humain mortel d'infection à *Chromobacterium violaceum* (septicémie et multiples abcès hépatiques). *Bull. Soc. Pat. exot.* **48**, 704.

DAVIES, D. A. L. (1955). The specific polysaccharides of some Gram-negative bacteria. *Biochem. J.* **59**, 696.

DODD, M. C. (1941). *Attempts to infect laboratory animals with strains of* Bacillus violaceus *isolated from human beings.* Dissertation, University of Michigan (University Microfilm Publications No. 319), 104 pp.

FILDES, P. (1920). A new medium for the growth of *B. influenzae. Brit. J. exp. Path.* **1**, 129.

FLOCH, H. & DE LAJUDIE, P. (1943). Sur le *Chromobacterium violaceum* et son pouvoir pathogène chez les animaux et chez l'homme. *Institut Pasteur de la Guyane et du Territoire de l'Inini* (*Archives de l'Institut Pasteur de la Guyane Française*), Publication No. 63.

GAUDUCHEAU, M. A. (1907). Sur un bacille violet pathogène. *C.R. Soc. Biol., Paris*, **62**, 278.

GILMAN, J. P. (1953). Studies on certain species of bacteria assigned to the genus *Chromobacterium. J. Bact.* **65**, 48.

HARTLEY, P. (1922). The value of Douglas's medium for the production of diphtheria toxin. *J. Path. Bact.* **25**, 479.

JOUBERT, L. & NGUYEN-VAN-LIEM (1957). La chromobactériose animale et humaine. *Rev. Elev.* n.s. **10**, 341.

LEIFSON, E. (1956). Morphological and physiological characteristics of the genus *Chromobacterium. J. Bact.* **71**, 393.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

MINETT, E. P. (1911). The frequency of *Bacillus violaceus* in the water and milk supplies of British Guiana. *Brit. Guiana med. Annu.* No. 18 (1913), p. 44.

NGUYEN-BA-LUONG (1956). A propos d'une épizootie porcine de mélioidose dans une province méridionale du Vietnam. *Bull. Soc. Pat. exot.* **49**, 25.

SCHATTENBERG, H. J. & HARRIS, W. H. (1942). *Chromobacterium violaceum* var. *manilae* as a pathogenic microorganism. *J. Bact.* **44**, 509.

SIPPEL, W. L. (1955). *A disease of swine caused by a* Chromobacterium *species.* Ph.D. Dissertation (Veterinary Pathology), Agricultural and Mechanical College of Texas, pp. 9–15, 63–67.

SIPPEL, W. L., MEDINA, G. & ATWOOD, M. B. (1954). Outbreaks of disease in animals associated with *Chromobacterium violaceum.* I. The disease in swine. *J. Amer. vet. med. Ass.* **124**, 467.

SNEATH, P. H. A. (1956*a*). Cultural and biochemical characteristics of the genus *Chromobacterium. J. gen. Microbiol.* **15**, 70.

SNEATH, P. H. A. (1956*b*). The change from polar to peritrichous flagellation in *Chromobacterium* spp. *J. gen. Microbiol.* **15**, 99.

SNEATH, P. H. A. (1957). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

SNEATH, P. H. A., WHELAN, J. P. F., SINGH, R. B. & EDWARDS, D. (1953). Fatal infection by *Chromobacterium violaceum. Lancet*, ii, 276.

WOOLLEY, P. G. (1904). Report on *Bacillus violaceus Manilae*: a pathogenic microorganism. *Bull. Bur. Labs Phil. Is.* No. 15.

WOOLLEY, P. G. (1905). *Bacillus violaceus Manilae* (a pathogenic microorganism). *Johns Hopk. Hosp. Bull.* **16**, 89.

WORLD HEALTH ORGANIZATION (1954). Expert committee on Biological Standardization: report of 7th Session, 1953. *Wld Hlth Org. tech. Rep. Ser.* No. 86, p. 14.

(*Received* 24 *October* 1958)

Elliott, S. D. & Barnes, Ella M. (1959). *J. gen. Microbiol.* **20**, 426–433

Changes in Serological Type and Antibiotic Resistance of Lancefield Group D Streptococci in Chickens Receiving Dietary Chlortetracycline

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SUMMARY: The effect of dietary chlortetracycline on the serological type and resistance of the streptococci found in the caeca of chickens has been investigated. In the control birds where only chlortetracycline-sensitive streptococci were present initially, *Streptococcus faecium* predominated and *S. faecalis* (mainly the proteolytic variant) was present in small numbers representing three serological types (types H69D5, D15 and D76). Administration of chlortetracycline, whether at a low concentration throughout life or intermittently at a high concentration, led to the emergence of a highly-resistant non-proteolytic strain of *S. faecalis* (type H69D5) which became predominant in the antibiotic treated birds. Withdrawal of chlortetracycline was followed by disappearance of type H69D5 and the reappearance of types D15 and D76 which were proteolytic. Type D15 remained sensitive to chlortetracycline, but type D76 had become more resistant and eventually became the predominant streptococcus in all the chickens under observation. The serological types of *S. faecalis* identified in these chickens are also commonly found in the human intestine.

Barnes (1958) reported the results of a survey of the intestinal streptococci of chicken and the effect of orally administered chlortetracycline on these micro-organisms. The streptococci belonged to *Streptococcus faecalis* (or a proteolytic variant *liquefaciens*) and *S. faecium*, two subgroups in Lancefield's group D, distinguishable by their biochemical characteristics. These streptococci were sensitive to chlortetracycline. Inclusion of this antibiotic in the food of the chickens was followed by an increased proportion of *S. faecalis* in the intestinal bacterial flora and these streptococci were highly resistant to chlortetracycline. Withdrawal of the antibiotic was followed by disappearance of the highly-resistant non-proteolytic streptococci and their replacement by a proteolytic variant of *S. faecalis*, of resistance intermediate between that of the original population and the population established during administration of the antibiotic.

The streptococci isolated during this survey and identified as *Streptococcus faecalis* have now been examined serologically with the object of determining whether changes in resistance reflected changes in serological type. Throughout this report the use of the terms 'liquefaciens' and 'zymogenes' as designating the proteolytic and haemolytic variants of *S. faecalis* has been avoided. These properties (particularly haemolysis) are of uncertain stability and it is clear that they may vary independently of the type-specific antigens. The name *S. faecalis* as used here therefore embraces all such group D streptococci as conform to the criteria suggested for this subgroup by Skadhauge (1950) and summarized by Barnes (1956), regardless of their haemolytic and proteolytic properties.

METHODS

Full details of the rearing and feeding of the chicken, the collection and examination of caecal and faecal samples, identification of the faecal streptococci and tests for their antibiotic resistance were given by Barnes (1958). Cultures classified as *Streptococcus faecalis* were further examined by using the methods described by Barnes, Ingram & Ingram (1956); their proteolytic properties were tested by their ability to liquefy gelatin.

The serological typing of group D streptococci

Lancefield (1941) identified three serological types among her group D streptococci and has since added one further type (personal communication); the type-specific antigens were thought to be polysaccharides. Sharpe & Shattock (1952) differentiated nine serological types, including Lancefield's original three types, among streptococci classified by them as *Streptococcus faecalis*; the type-specific antigens were not characterized chemically but were recognized by agglutination and precipitation reactions. In the work to be described here the serological types of *S. faecalis* were characterized antigenically by their cell-wall polysaccharides. Evidence that in group D streptococci the type-specific polysaccharides are an integral part of the bacterial cell wall will be presented by one of us (S.D.E.) later. They can be extracted from the streptococci either by tryptic digestion or by treatment with hot acid, and identified by precipitin tests with hyperimmune rabbit serum of homologous type. The immunization of rabbits with formalin-killed group D streptococci commonly leads to the production of type-specific antibody but little, if any group-specific antibody. Serum taken from rabbits in the early stages of immunization often gives only type-specific precipitation reactions especially when, as is the case with tryptic digests, the bacterial extracts contain minimal amounts of group antigen. In the present work, the 'typing' of streptococci was accomplished by precipitin tests with tryptic digests and with acid extracts as the source of type-specific antigen. The antigen preparations were tested first against crude antisera and positive reactions subsequently confirmed with type-specific sera from which group antibody had been removed by absorption with strains of heterologous type.

Preparation of type-specific antisera. Type-specific precipitating antisera were prepared in rabbits against Lancefield's 4 group D 'type' strains (strains D76, H69D5, C1, C3) and also against two strains of *Streptococcus faecalis* (D10 and D15) isolated by Evans (1957) from cases of bovine endocarditis and later shown to be distinct serological types in group D (Elliott, 1957). Strain C3 belongs to *S. durans*; strains D76, H69D5, C1 and D15 belong to

S. faecalis and correspond to types 1, 3, 4, and 9 in the classification of Sharpe & Shattock (1952).

Vaccines were prepared from streptoccci grown in Todd-Hewitt broth at room temperature for 18 hr. and killed by the addition of formaldehyde (final concentration 2%, w/v). After 2–3 days the cocci were washed and resuspended in saline. Procedures for the immunization of rabbits and absorption of antisera were those recommended by Lancefield (1938, 1940). For absorbing non-specific antibody from group D typing sera Lancefield's strain C1 was generally used. Most sera required several absorptions before they became type specific and in some instances those strains which gave persistent cross-reactions were used for the final absorption.

Preparation of streptococcal extracts. (i) Tryptic digestion. Cultures were grown in 40 ml. amounts of Todd-Hewitt broth for 18 hr. at room temperature. Formaldehyde was then added (final concentration 2%, w/v) and the cultures left at room temperature for 24–48 hr. The streptococci were then washed, first in saline and then in M/30 phosphate buffer (pH 8). Finally, they were suspended in 0·5 ml. phosphate buffer (pH 8) containing crystalline trypsin 0·1 mg./ml. and incubated for 1 hr. at 37°. After this digestion, the supernatant fluid contained mainly type antigen and little, if any, group-specific antigen and was used in precipitation reactions without further treatment. (ii) Acid extraction. The method of Lancefield (1940) was used. Streptococci from 40 ml. cultures which had first been subjected to tryptic digestion (see above) were subsequently resuspended in a volume of 0·5 ml. and heated at 100° and *c.* pH 2 for 10 min. Extracts prepared by this method contained type and group antigens.

Immunological reactions. The precipitin test by the capillary technique was used (Swift, Wilson & Lancefield, 1943). Capillary tubes (internal diam. 1·0 mm.) were used and the 'typing' sera were such that positive reactions could be read within 15 min. at room temperature.

RESULTS

Effect of a dietary supplement of chlortetracycline on Streptococcus faecalis *in the intestine of chickens*

The design of this experiment was described by Barnes (1958). Three groups of chickens were reared under conditions which were identical except that two groups received a dietary supplement of chlortetracycline: one group received the control ration with a constant supplement of chlortetracycline at 25 p.p.m.; the other received an intermittent supplement at 400 p.p.m. At the end of 12 weeks, 12 birds in each group were killed and the relative numbers of *Streptococcus faecalis* and *S. faecium* in their caeca determined. In 9 of the control birds and 8 of those which received chlortetracycline a more detailed bacteriological examination was made. From the control birds 19 colonies of *S. faecalis* (1 to 4 from each bird), and from the birds which received chlortetracycline 53 colonies (5 to 10 from each), were serologically typed. The results are shown in Table 1.

It will be seen that in the control birds *Streptococcus faecium* was the predominant streptococcus. *S. faecalis* was present only in very small numbers; indeed, it was difficult to obtain a sufficient number of colonies for further bacteriological examination. None of these streptococci were able to grow on nutrient agar + chlortetracycline at 10 p.p.m. Extracts prepared from 17 strains of *S. faecium* (from 8 birds) failed to react with any of the five *S. faecalis* typing sera available, three of which, corresponding to types D76, D15 and H69D5, were sufficient to identify all 19 cultures of *S. faecalis* isolated from the control birds. Of the 13 control cultures belonging to type D76 or to D15, all were proteolytic, whereas of 6 belonging to type H69D5, 2 were proteolytic and 4 non-proteolytic.

Table 1. *Effect of dietary chlortetracycline on faecal streptococci in the caeca of chickens at* 12 *weeks*

			No. of birds with *S. faecalis* of specified serological type, CTC* resistance and proteolytic activity				
			Proteolytic			Non-proteolytic	
Diet	Approx. ratio: *S. faecalis*/*S. faecium*	No. of birds (or colonies) examined in detail	Type D76 sensitive	Type D15 sensitive	Type H69D5 sensitive	Type H69D5 sensitive	Type H69D5 resistant†
Control	0·001	9 (19)	1 (2)	5 (11)	1 (2)	4 (4)	—
Continuous CTC supplement 25 p.p.m.	1·0	6 (38)	—	—	—	—	6 (38)
Intermittent CTC supplement 40 p.p.m.	100	2 (15)	—	—	—	—	2 (15)

( ) = Number of colonies examined.
* CTC = Chlortetracycline.
+ Sensitive = failed to grow on agar containing CTC 10 p.p.m.
† Resistant = grew on agar containing CTC 200 p.p.m.

The caecal cultures from the two groups of birds which received the chlortetracycline supplement were in striking contrast to those from the control group. In the birds which received the small but continuous supplement (25 p.p.m.) the numbers of *Streptococcus faecalis* and *S. faecium* were approximately equal, while in those birds given the larger intermittent supplement (400 p.p.m.), *S. faecalis* was predominant. All cultures of *S. faecalis* isolated from either group grew on nutrient agar + chlortetracycline at 200 p.p.m.; all were non-proteolytic; and all belonged to one serological type, type H69D5. It is noteworthy that non-proteolytic type H69D5 streptococci were present in four of the control birds but these were not able to grow on nutrient agar + chlortetracycline at 10 p.p.m. By using the serial dilution technique with Difco Penassay broth containing chlortetracycline it was confirmed that the type H69D5 cultures isolated from the birds fed chlortetracycline were about 200 times more resistant than those of the same serotype recovered from control birds. From the results of this experiment it was concluded that oral administration of chlortetracycline led to the development of a resistant variant of *S. faecalis* type H69D5. This variant may have appeared originally in one bird only or simultaneously in two or more but, through selective proliferation in the presence of the antibiotic, it supplanted most of the other streptococci in the intestines of those chickens receiving chlortetracycline.

Effect of withdrawing the chlortetracycline dietary supplement on Streptococcus faecalis *in the intestinal tract of chickens*

The effect of withdrawing chlortetracycline from the diet was tested on those birds which had previously received the larger supplement (400 p.p.m.), since they showed the greatest difference in streptococcal population from the control group. The 38 surviving birds (12 weeks old) in both the control and chlortetracycline groups were given the same diet (without antibiotic) during 5 months. At the end of 3 months bulk samples of intestinal dejecta from each group were examined. After a further 2 months, i.e. 5 months after discontinuing the antibiotic supplement, three birds from the group originally given chlortetracycline and two from the control group were killed. Bacteriological examinations of the caecal contents were made as before and colonies of *Streptococcus faecalis* isolated for further study.

Table 2. *Change in faecal streptococci in chickens following withdrawal of a dietary supplement of chlortetracycline at* 12 *weeks of age*

				No. of colonies of specified serological type		
				Proteolytic		Non-proteolytic
Age at sampling	Source of sample and no. of birds examined	Approx. ratio *S. faecalis* / *S. faecium*	No. of colonies of *S. faecalis* examined in detail	Type D15 sensitive*	Type D76 resistant†	Type H69D5 resistant‡
12 weeks	Caeca, 2 birds	100	15	—	—	15
6 months	Bulk faecal samples, 38 birds	10 (100)	24 (6)	21	3 (4)	—
8 months	Caeca, 3 birds	5 (10)	25 (28)	1	24 (28)	—

( ) = Corresponding data from control birds which had never received chlortetracycline (CTC).

* Sensitive = failed to grow on agar containing CTC 10 p.p.m.

† Resistant = grew on agar containing CTC 50 p.p.m.

‡ Resistant = grew on agar containing CTC 200 p.p.m.

The results of these surveys are shown in Table 2. It will be seen that in the 6 months old birds i.e. 3 months after discontinuing the antibiotic supplement, *Streptococcus faecalis* outnumbered *S. faecium* in the dejecta of control and of previously antibiotic-fed birds. Type H69D5 streptococci were not found in either group, but a chlortetracycline-resistant proteolytic strain of type D76 appeared in both. Type D15, previously found in the control group and presumably present (although not demonstrated) in the antibiotic treated birds, had now reappeared in the latter and remained sensitive to chlortetracycline. Two months later, i.e. 5 months after discontinuing the antibiotic supplement, the chlortetracycline-resistant type D76 had become established as the predominant streptococcus in the intestinal tract of all the birds examined. Type D15, sensitive to chlortetracycline, was still present in at least one chicken, but the type H69D5 streptococcus was not found in any.

DISCUSSION

Of the 155 cultures of *Streptococcus faecalis* examined in the course of this investigation all except two were identified as belonging to one or other of three distinct serological types. It is of interest that we have also identified these types among streptococci isolated from adult human intestinal tracts; the same types were found by Sharpe & Shattock (1952) in infants with neonatal diarrhoea. The small number of types represented in the present study might be thought to detract from the validity of conclusions based on the assumption that organisms belonging to the same type represent a single strain and had a common origin. For example, it might be doubted whether the type D15 streptococci encountered in birds from whose diet chlortetracycline had been withdrawn were the direct descendants of streptococci of the same type encountered in controls and presumably present, although not demonstrated, in all birds before administration of the antibiotic. It will be recalled that during the intervening period, i.e. during administration of chlortetracycline, type D15 disappeared and its place was taken by type H69D5. Similarly, in the absence of additional evidence, it could be open to question whether the chlortetracycline-resistant type H69D5 streptococci were developed from the sensitive micro-organisms of this type present before administration of the antibiotic. Our belief that such was the case is strengthened by the existence of independent 'strain markers' characterizing these streptococci of type D15 and type H69D5. All of the cultures identified as type D15 were haemolytic when grown on horse blood agar and, apart from these cultures, no other haemolytic strain was encountered during this investigation. Non-haemolytic strains of type D15 had previously been found in other birds in the same locality. Similarly, all but two of the cultures identified as type H69D5 were non-proteolytic and these were the only non-proteolytic cultures of *S. faecalis* isolated from the control birds. Indeed, non-proteolytic strains of *S. faecalis* are rarely met with in the intestinal tract of chickens fed on a normal diet.

It is our impression that under the conditions of antibiotic feeding imposed in this experiment, chlortetracycline-sensitive streptococci originally present in the intestinal tract of the chickens were suppressed but not completely eliminated. Their place was taken by a strain of *Streptococcus faecalis* type H69D5 which rapidly developed resistance to the antibiotic. That this resistant strain was non-proteolytic may have been fortuitous. Alternatively, it is possible that resistance to chlortetracycline is more readily developed by non-proteolytic than by proteolytic streptococci of this type, for it will be recalled that both proteolytic and non-proteolytic chlortetracycline-sensitive type H69D5 streptococci were present in the control birds at the beginning of the experiment. Supporting this hypothesis were the results of an *in vitro* experiment in which the sensitive proteolytic and non-proteolytic type H69D5 streptococci were serially subcultivated in broth containing increasing concentrations of chlortetracycline. Under these conditions the resistance of the non-proteolytic variant increased 32-fold while that of the proteolytic variant increased only eightfold. With both cultures considerable difficulty was experienced in further increasing their resistance.

It is of interest that from the birds fed throughout life with a 25 p.p.m. chlortetracycline supplement and from those which received an intermittent 400 p.p.m. supplement an identical highly resistant type H69D5 streptococcus was isolated. The two groups of birds differed in that, although the total number of faecal streptococci was approximately the same in both, in the group which received the larger supplement type H69D5 streptococci markedly outnumbered *Streptococcus faecium* whereas in the birds given the smaller supplement, type H69D5 and *S. faecium* were present in equal numbers. It may well be that the wide distribution of the resistant type H69D5 streptococcus in the birds which received chlortetracycline was due to cross infection. The degree to which this micro-organisms outnumbered the less resistant faecal streptococci clearly depended, in part, upon the concentration of the antibiotic in the intestines.

Following withdrawal of the antibiotic, conditions in the intestinal tract reverted to normal and presumably became more favourable for the proliferation of proteolytic than for non-proteolytic streptococci, for the former promptly regained the ascendancy and the non-proteolytic chlortetracycline-resistant strain disappeared. Finally, a single proteolytic strain, type D76, which had developed some degree of resistance to chlortetracycline, became established as the predominant representative of *Streptococcus faecalis* in the intestinal tract of control chicken and of those previously fed the antibiotic. The reason for the final ascendancy of this particular strain is not clear. Its resistance to chlortetracycline would hardly be advantageous in the absence of the antibiotic; R. F. Gordon & J. F. Tucker (personal communication) also found a significant proportion of resistant coliforms in these chickens 5 months after the antibiotic supplement was withdrawn.

We are indebted to Dr R. C. Lancefield for type-specific antisera corresponding to her 4 group D 'type' strains. The contribution of E.M.B. to this paper was as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Barnes, E. M. (1956). Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. *J. gen. Microbiol.* **14**, 57.

Barnes, E. M. (1958). The effect of antibiotic supplements on the faecal streptococci (Lancefield group D) of poultry. *Brit. vet. J.* **114**, 333.

Barnes, E. M., Ingram, M. & Ingram, G. C. (1956). The distribution and significance of different species of faecal streptococci in bacon factories. *J. appl. Bact.* **19**, 204.

ELLIOTT, S. D. (1957). Bacterial endocarditis of cattle. *Vet. Rec.* **69**, 1203.

EVANS, E. T. REES (1957). Bacterial endocarditis of cattle. *Vet. Rec.* **69**, 1190.

LANCEFIELD, R. C. (1938). A micro precipitin technique for classifying haemolytic streptococci and improved methods for producing antisera. *Proc. Soc. exp. Biol., N.Y.* **38**, 473.

LANCEFIELD, R. C. (1940). Type-specific antigens, M and T of matt and glossy variants of group A haemolytic streptococci. *J. exp. Med.* **71**, 521.

LANCEFIELD, R. C. (1941). Specific relationship of cell composition to biological activity of haemolytic streptococci. *Harvey Lect.* Series 36, 251.

SHARPE, M. E. & SHATTOCK, P. M. F. (1952). The serological typing of group D streptococci associated with outbreaks of neonatal diarrhoea. *J. gen. Microbiol.* **6**, 150.

SKADHAUGE, K. (1950). *Studies on Enterococci with Special Reference to the Serological Properties.* Copenhagen: Einar Munksgaard.

SWIFT, H. F., WILSON, A. T. & LANCEFIELD, R. C. (1943). Typing group A haemolytic streptococci by M precipitin reactions in capillary pipettes. *J. exp. Med.* **78**, 127.

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Mitchell, P. & Moyle, J. (1959). *J. gen. Microbiol.* 20, 434–441

Permeability of the Envelopes of *Staphylococcus aureus* to some Salts, Amino Acids, and Non-Electrolytes

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SUMMARY: The passive permeability properties of the plasma-membrane of *Staphylococcus aureus* (strain Duncan) resemble those of a classical lipid membrane such as that considered by Overton in 1899. In general, solutes carrying more than four water molecules flow across the plasma-membrane only very slowly under an electrochemical gradient. The plasma-membrane is the effective osmotic barrier to small molecular weight solutes and prevents the escape of internal components having a total osmotic concentration corresponding to *c.* 1 molal sucrose. The protoplast is prevented from swelling by the cell wall which withstands a hydrostatic thrust of some 20 to 30 atmospheres pressure exerted against it by the plasma-membrane in distilled water. The pores in the cell wall, although large enough to permit rapid diffusion of small molecular weight solutes, are too small to allow a dextran of mol. wt. 10,000 to penetrate. The cell wall acts as the osmotic barrier for large molecular weight components, preventing such components in the medium from gaining access to the plasma-membrane surface and preventing such internal components from passing outwards from the surface of the plasma-membrane.

In this paper we have collected together observations made at intervals during the last four years on the permeability of the envelopes of *Staphylococcus aureus* (strain Duncan). Part of this work was done in the Biochemistry Department, University of Cambridge.

'*Permeability.*' The word permeability as used in this paper is an index of the rate at which a given solute undergoes *net transport* through the plasma-membrane under an electrochemical gradient. For reasons which have been discussed elsewhere (Mitchell & Moyle, 1956*b*), permeability in this sense must not be confused with the property of a membrane which may allow exchange of a solute across it without net transport. However, while we wish to restrict the meaning of permeability here to the description of net transport, we do not wish to restrict it to the sense given by the permeability coefficient as defined by a law of the Fick type, because we do not know whether the rates of solute diffusion which we describe in this paper are linear functions of the concentration or electrochemical gradient as they would have to be if they were described by a Fick law.

Plasma-membrane. The word plasma-membrane is used to describe the outer region of the protoplasm of the bacterium which is normally in contact with the inner surface of the cell wall. This region consists of a lipo-protein sheet, *c.* 5 mμ thick containing some 20 % lipid and 40 % protein (Mitchell & Moyle, 1951). Comparison of the phosphate-impermeable volume of *Staphylococcus aureus* with the volume of the protoplast calculated from the geometry of close-packed suspensions of the cocci indicates that the osmotic barrier for phosphate is some part of the plasma-membrane (Mitchell, 1953). Similar considerations, applied to intact organisms and to protoplasts freed from the mechanical protection of the cell wall, imply that the osmotic barrier for small molecular weight solutes is some part of the plasma-membrane in *Bacillus megaterium* (Weibull, 1955), *Micrococcus lysodeikticus* and *Sarcina lutea* (Mitchell & Moyle, 1956*a*, *c*), and several strains of *Staphylococcus aureus* and *albus* (Mitchell & Moyle, 1957).

In the present paper we shall describe the plasma-membrane of *Staphylococcus aureus* by its functions (i) of retaining internal solutes, and (ii) of excluding external solutes. Function (i) may be assessed from the internal osmotic pressure measured either by the osmotic pressure required to prevent swelling and lysis of cell-wall-free protoplasts, as already described (Mitchell & Moyle, 1956*c*, 1957); or by a vapour pressure equilibrium method applicable to intact organisms (Mitchell & Moyle, 1956*a*) which we shall now describe in full. Function (ii) may be assessed by measurements of the rate of penetration of solutes into the cells of very thick suspensions of organisms, or by measurements of the rate of swelling and lysis of protoplasts suspended in isotonic or hypertonic solutions of the solute to be studied.

METHODS

Growth and preparation of organisms. Staphylococcus aureus (strain Duncan) was grown, harvested and washed as previously described by Mitchell (1953) and by Mitchell & Moyle (1957).

Osmotic pressure measurements by vapour pressure equilibrium. A washed suspension of *Staphylococcus aureus*, harvested near the stationary phase of growth (2 mg. dry weight/ml.), containing *c.* 1 g. dry-weight organisms was centrifugally concentrated to give a just pipettable suspension of some 250 mg. dry weight/ml. Samples (0·2 ml.) of this suspension were carefully but rapidly extruded (taking *c.* 60 sec.) from a 0·2 ml. pipette on to the centre of pieces of polythene 4 cm. square so as to form circular films 1·5 cm. in diameter, and each was immediately covered by a watch-glass to prevent evaporation. The cell samples were then weighed to within the nearest 0·1 mg. and were transferred to small desiccators, each containing a dish of aqueous sucrose solution (10 ml.) of known molality, close to the surface of which (*c.* 2 mm.) the polythene squares could be placed with the film of organisms downwards. The desiccators were simultaneously evacuated to a pressure of 2 mm., measured by a McLeod gauge, and vapour equilibration was allowed to proceed at 20°. In order to prevent air bubbles from splashing droplets of the sucrose solutions on to the polythene films, it was found to be necessary to de-gas the sucrose solutions by a preliminary period of evacuation. It was found that vapour equilibrium was practically complete in 5 hr. under the conditions of these experiments, and the films of organisms were accordingly reweighed after 5 hr. of equilibration. A longer period of equilibration was not desirable as it was important to minimize lysis and other changes which might occur to the organisms. The dry weight at zero vapour pressure was found by equilibrating films over phosphorus pentoxide *in vacuo*. From these observations the weight of water retained/unit weight organisms could be related to the equilibrium water vapour pressure of equivalent osmotic pressure.

Permeability measurements on thick suspensions. The basic methods used in handling the suspensions were as previously described (Mitchell, 1953). The permeability measurements with glutamate, glutamine, glycine, and a Leuconostoc dextran of mol. wt. 10,000 were done at pH 7 in 0·1 M-NaCl and 0·01 M-sodium phosphates. Glutamic acid and glutamine were measured by the decarboxylase method (Gale, 1945), and glycine was measured by oxidation with triketohydrindene hydrate, distillation of the formaldehyde produced and colorimetric estimation with chromotropic acid (Alexander, Landwehr & Seligman, 1945). The dextran was measured by the anthrone reaction as described by Mitchell & Moyle (1951).

The permeability measurements on chloride and thiocyanate were done with an initial concentration of 0·1 M-sodium salts in the presence of 0·01 M-sodium phosphates at pH 7·2. Chloride and thiocyanate were estimated volumetrically by adding excess silver nitrate and back-titrating with thiocyanate in dilute nitric acid, with ferrous ammonium sulphate as indicator. The volume of the cells impermeable to phosphate was determined at the same time as the volume impermeable to the other solutes, as a check on the variations in cell size that were found to occur from one culture to another.

Permeability measurements by 'protoplast' lysis. The protoplasts were released from the mechanical protection of the cell wall, and the extent of lysis of the 'protoplast' suspensions was measured by light extinction as previously described (Mitchell & Moyle, 1957). The permeability measurements were done at 20° in 1·5 molal solutions containing 0·01 M-sodium phosphates at pH 6·8.

RESULTS AND CONCLUSIONS

Figure 1 shows the results of the vapour pressure equilibrium measurements, plotted as the weight of water/g. dry weight organisms against the reciprocal of the molality of the equilibrating sucrose solutions. Since the behaviour of sucrose solutions is nearly ideal, it can be taken that the reciprocal of the molality of the equilibrating sucrose solution is proportional to the reciprocal of the difference between the equivalent osmotic pressure and the hydrostatic pressure of the cytoplasm of the cells at equilibrium. In an ideal solution (in which the solute molecules move independently of each other and of the molecules of solvent) the reciprocal of the osmotic pressure is proportional to the weight (or volume) of solvent. It will be seen in Fig. 1 that the weight of water held in the organisms was proportional to the reciprocal of the osmotic pressure (or to 1/molality of sucrose) for values up to 1·8 g. water/g. dry weight organisms, corresponding to an osmotic pressure of 1 molal sucrose. But, above this value, the weight of water held by the organisms increased much more slowly with the reciprocal of the osmotic pressure of the equilibrating sucrose solutions. The weight of water held by the organisms at the upper limit of the linear range of the graph (1·8 g. water/g. dry weight organisms) was in reasonably good agreement with the volume of water in the protoplasts estimated directly to be 1·5–1·6 ml./g. dry-weight organisms (Mitchell, 1953). Probably the additional 0·2–0·3 g. water found by the vapour-pressure equilibrium measurements was held by hydrophilic components of the cell wall. The position of the discontinuity in the graph implies that the cell wall resisted the dilution of the solutes contained within the protoplast beyond a content of 1·8 g. water/g. dry weight organisms. As the equilibrating vapour pressure (or 1/molality of sucrose) was raised above this point, the elasticity of the cell wall would be taken up and the internal osmotic pressure, exerted against the plasma-membrane, would be compensated by the hydrostatic pressure of the cell wall, so that the equilibrium vapour pressure would correspond to that of the water outside the organisms. This water would contain a little solute due, presumably, to a small degree of cell lysis or leakage. We may conclude that the normal osmotic pressure of the cytoplasm of these organisms corresponded approximately to 1 molal sucrose or to some 20 to 25 atmospheres, in good agreement with the value obtained by measurements of the dependence of the swelling and lysis of the 'protoplasts' of this organism on the tonicity of the suspension media (Mitchell & Moyle, 1957).

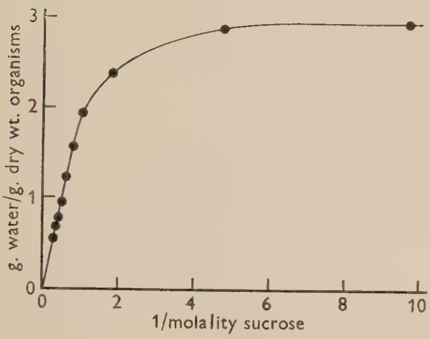

Fig. 1

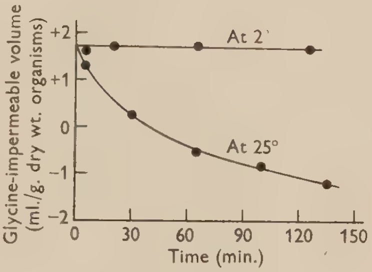

Fig. 2

Fig. 1. Vapour pressure equilibrium data for *Staphylococcus aureus* (strain Duncan), given as g. water retained/g. dry wt. organisms in equilibrium with sucrose solutions of known molality at 20°.

Fig. 2. Time course of glycine-impermeable volume of *Staphylococcus aureus* (strain Duncan) at 2° and 25°.

Table 1 shows the volume of *Staphylococcus aureus* impermeable to glutamic acid, glutamine, glycine, chloride, thiocyanate and a Leuconostoc dextran (mol.wt.10,000) at ionic strength 0·1 and close to neutrality. The corresponding phosphate-impermeable volumes are also given for comparison since, as will be seen, the volume of the organisms varied somewhat from one batch to another. The values in brackets were obtained after treating the organisms with 5 % (w/v) *n*-butanol in water for 10 min. at 2°. The last column shows the amount of the solutes which appeared to pass in through the osmotic barrier, assuming the position of this barrier to be given by the phosphate-impermeable

Table 1. *Solute-impermeable volumes of* Staphylococcus aureus (*strain Duncan*)

Values in brackets refer to cocci treated with 5 % (w/v) *n*-butanol in water

Solute in suspenison medium (approx. initial concn.)	Time and temp. of equilibration	Solute-permeable vol./g. dry wt. organisms	Phosphate-impermeable vol./g. dry wt. organisms	Mg. dry wt. organisms/ml. suspension	μmole solute disappearing from normal external phosphate space/g. dry wt. organisms
Glutamic acid (0·02 M)	5 min., 25°	2·25	2·34	203	1·7
Glutamine (0·02 M)	5 min., 25°	2·01			6·0
Glutamic acid (0·02 M)	5 min., 2°	1·93	1·91	242	0·4
Glutamic acid (0·02 M)	30 min., 25°	1·87			0·8
Glutamine (0·02 M)	5 min., 2°	1·55			6·4
Glutamine (0·02 M)	30 min., 25°	1·51			7·2
Glutamic acid (0·04 M)	5 min., 2°	1·87		230	1·5
Glutamic acid (0·04 M)	30 min., 25°	1·89			0·7
Glutamine (0·04 M)	5 min., 2°	1·76			5·1
Glutamine (0·04 M)	30 min., 25°	1·72			6·2
Glycine (0·02 M)	5 min., 2°	1·60 (−0·46)	2·17 (0·80)	235	8·9 (23·4)
Glycine (0·02 M)	30 min., 25°	−2·38 (−2·62)			29·3 (29·6)
Glycine (0·04 M)	5 min., 2°	1·86 (−0·11)			10·6 (41·3)
Glycine (0·04 M)	30 min., 25°	−0·25 (−1·12)			45·7 (41·1)
Chloride (0·10 M)	5 min., 2°	1·89	2·07	233	17
Chloride (0·10 M)	35 min., 2°	1·76			27
Thiocyanate (0·10 M)	5 min., 2°	1·50			45
Thiocyanate (0·10 M)	30 min., 2°	1·11			67
Dextran (1 % w/v)	30 min., 25°	2·39 (2·15)	2·21 (0·78)	230	—

volumes: the figures may represent either permeation or adsorption. It will be observed that neither glutamic acid nor glutamine penetrated the osmotic barrier to a significant extent during 30 min. at 25°, but, whereas glutamic acid behaved almost exactly like inorganic phosphate, some 5–7 μmole glutamine disappeared from the external phosphate space within 5 min. (the time required for centrifugation) at 2°. Since the amount of glutamine which disappeared was approximately the same whether the external concentration was 0·02 or 0·04 M, and the amount increased very little in 5 or 30 min. at 25°, it seems probable that this glutamine was adsorbed on the cell wall or on the surface of the osmotic barrier. The value for the glycine-impermeable volume is similar to that for glutamine at 2°, but at 25° it rapidly becomes negative. This means that the amount of glycine which disappeared from the suspension medium was greater than can be accounted for by completely uniform distribution even including the space that must be occupied by the substance of the organisms. The organisms therefore appeared to occupy a negative volume. On adding butanol to give a 5% solution so as to break the osmotic barrier of the cells, an amount of glycine was taken up from the medium which was near to that taken up by intact organisms in 30 min. at 25°, even in the suspensions held at 2° (values in brackets, Table 1). The results indicate that at 2° the organisms were impermeable to glycine, but that some 10 μmole glycine was adsorbed outside the osmotic barrier. When the organisms were treated with butanol or when the temperature was raised to 25° the glycine evidently passed through the osmotic barrier. Since the cell materials must occupy some 0·8 ml./g. dry weight organisms, the total amounts of glycine which disappeared when the osmotic barrier was broken with butanol at 2° were 15 and 24 μmole/g. dry weight organisms when the final glycine concentrations were, respectively, 8 and 18 mM. It is unlikely that this glycine was metabolized at 2° in the presence of 5% butanol, and hence it was probably adsorbed, partly in the surface (where it adsorbs before penetration) and partly within the cells. This is in accord with Gale's observation of a spontaneous uptake of radioactive glycine by this organism (Gale, 1953). In order to throw more light on the relative rates of permeation of glycine at 2° and at 25°, the time course of the glycine-impermeable volume was measured, with an initial glycine concentration of 0·04 M (Fig. 2). At 2°, glycine did not permeate significantly in 2 hr., but at 25° half-equilibration occurred in *c.* 30 min. The fact that glycine permeation has a very high temperature coefficient suggests that it may involve a specific carrier mechanism.

Judging from the values in Table 1, chloride entered the cells slowly, and thiocyanate entered rapidly at 2°. The chloride- and thiocyanate-impermeable volumes are plotted against time at 2° in Fig. 3, with phosphate for comparison. It appears as though *Staphylococcus aureus* is somewhat permeable to chloride, and very permeable to thiocyanate. The thiocyanate ion does not seriously damage the osmotic barrier at this concentration, for even M-NaCNS was found not to cause leakage of phosphate across the osmotic barrier. Since the anion and cation probably penetrate independently, these results suggest that Na^+ penetrates readily while the ease of penetration for the anions is in the order $CNS' > Cl' > (H_2PO_4' + HPO_4'')$. We cannot, however, exclude the possibility that thiocyanate artificially raises the permeability of the plasma-membrane to Na^+. The fact that the equilibration of Cl′ appeared to become complete when only some 0·5 ml. of the total protoplasmic water (*c.* 1·2 ml./g. dry weight organisms in this experiment) would have become accessible, suggests that either the chloride ion was exchanging stoichiometrically across the membrane for some other anion, or that the coccal suspension was not homogeneous and only some 40% of the cocci were permeable to chloride.

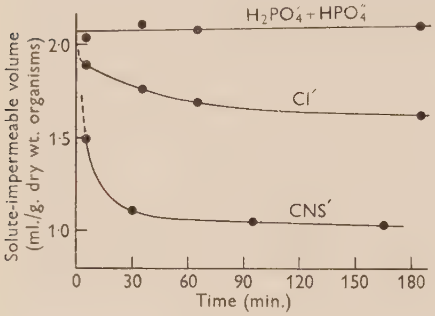

Fig. 3

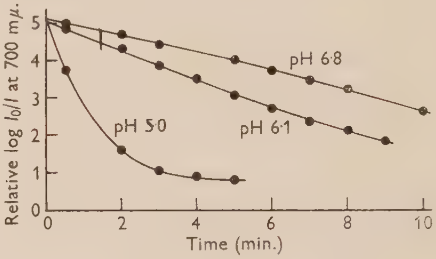

Fig. 4

Fig. 3. **Time-course of phosphate-, chloride-, and thiocyanate-impermeable volumes of *Staphylococcus aureus* (strain Duncan) at 2°.**

Fig. 4. **Time-course of permeation of KCNS into 'protoplasts' of *Staphylococcus aureus* (strain Duncan) at three pH values. 'Protoplasts' suspended in 1·5 molal KCNS**

Table 1 shows that the volumes impermeable to phosphate and to dextran in normal cocci were, respectively, 2·21 and 2·39 ml./g. dry weight organisms, while after treatment with butanol they were 0·78 and 2·15 ml./g. dry weight organisms. Thus, the osmotic barrier for phosphate is not the same as that for the relatively high molecular weight (10,000) dextran. The osmotic barrier for phosphate must be part of the plasma-membrane, while the osmotic barrier for the dextran (approaching from outside) is presumably the outer region of the cell wall.

The rate of lysis of the 'protoplasts' of *Staphylococcus aureus* is less than 10%/hr. at 20° in 1·5 molal solutions of the following solutes at pH 6·8 in the presence of 0·01 M-sodium phosphates, except where otherwise indicated: NaCl, KCl, NH_4Cl, KBr, KNO_3, sodium acetate (pH 9), potassium acetate (pH 9), K_2SO_4, $(KH_2PO_4 + K_2HPO_4)$, sodium glutamate, lysine hydrochloride, D-glucose, D-sorbose, D-mannose, D-galactose, D-fructose and sucrose. In KCNS, however, the 'protoplasts' break in the course of a few minutes as shown in Fig. 4, the rate of breakage being very sensitive to the pH value. In NaCNS, similar curves show a rate of lysis *c.* 0·8 of that observed in the corresponding KCNS solution. The more rapid lysis at lower pH values could not be due simply to an increase in the fragility of the 'protoplasts', for there was no lysis in NaCl solutions at corresponding pH values. These results are in accord with the observations described above on the permeability of intact

S. aureus to thiocyanate. The time for half-lysis of the 'protoplasts' of *S. aureus* is less than 3 sec. for glycerol, it is 20 sec. for erythritol, 5 min. for D-ribose, 30 min. for L-arabinose and 20 min. for D-sorbitol. The 'protoplasts' lyse almost instantaneously in 1·5 molal urea or glycine, but it is probable that these protein denaturants have a direct effect upon the fragility of the membrane.

It is evident that the permeability properties of the cells of *Staphylococcus aureus* freed from the mechanical protection of the cell wall are essentially similar to those of *Micrococcus lysodeikticus* and *Sarcina lutea*, and closely resemble those of intact *S. aureus*. The plasma-membranes of these organisms appear to exhibit the passive permeability properties expected of a classical lipid membrane like that originally considered by Overton in 1899.

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REFERENCES

ALEXANDER, B., LANDWEHR, G. & SELIGMAN, A. M. (1945). A specific micromethod for the colorimetric determination of glycine in blood and urine. *J. biol. Chem.* **160**, 51.

GALE, E. F. (1945). Studies on bacterial amino-acid decarboxylases. 5. The use of specific decarboxylase preparations in the estimation of amino-acids and in protein analysis. *Biochem. J.* **39**, 46.

GALE, E. F. (1953). Assimilation of amino-acids by Gram-positive bacteria and some actions of antibiotics thereon. *Advanc. Protein Chem.* **8**, 285.

MITCHELL, P. (1953). Transport of phosphate across the surface of *Micrococcus pyogenes*: nature of the cell 'inorganic phosphate'. *J. gen. Microbiol.* **9**, 273.

MITCHELL, P. & MOYLE, J. (1951). The glycerophosphoprotein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

MITCHELL, P. & MOYLE, J. (1956*a*). Osmotic function and structure in bacteria. In *Bacterial Anatomy*. *Symp. Soc. gen. Microbiol.* **6**, 150.

MITCHELL, P. & MOYLE, J. (1956*b*). Permeation mechanisms in bacterial membranes. *Disc. Faraday Soc.* **21**, 258.

MITCHELL, P. & MOYLE, J. (1956*c*). Liberation and osmotic properties of the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea*. *J. gen. Microbiol.* **15**, 512.

MITCHELL, P. & MOYLE, J. (1957). Autolytic release and osmotic properties of 'protoplasts' from *Staphylococcus aureus*. *J. gen. Microbiol.* **16**, 184.

OVERTON, E. (1899). Über die allgemeinen osmotischen Eigenschaften der Zellen, ihre vermutlichen Ursachen und ihre Bedeutung für die Physiologie. *Vjschr. naturf. Ges. Zürich*, **44**, 88.

WEIBULL, C. (1955). The localization of a permeability barrier in the cells of *Bacillus megaterium*. *Exp. Cell Res.* **9**, 139.

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Braendle, D. H., Gardiner, Beth & Szybalski, W. (1959). *J. gen. Microbiol.* **20**, 442–450

Heterokaryotic Compatibility in Streptomyces

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SUMMARY: The compatibility system in *Streptomyces fradiae* may be characterized as follows. When 10^8 conidia of each of two freshly isolated auxotrophic mutants are mated on minimal medium, a small number, usually 1 to 10, of 'primary' heterokaryotic colonies appear (low-frequency mating, LFM). On the other hand, when 'secondary parental isolates' (derived from the conidia produced by 'primary' heterokaryons) are mated, the frequency of heterokaryon formation is increased by a factor of several thousand. This high-frequency mating (HFM) is a reasonably stable property, decreasing (or increasing) in a stochastic manner, only after a considerable number of subcultures. A mutational origin is therefore postulated for the HFM and LFM isolates, which must be endowed with variable cultural fitness causing diverse population shifts upon subculture. More effective anastomosis, not increase in residual growth of the 'secondary parental isolates', appears to be responsible for HFM. No interspecific crosses were successful, even when an HFM tester stock was used, but heterokaryotic interaction between independently isolated auxotrophic mutants derived from the same line of *S. fradiae* seemed to increase when an HFM strain was one of the partners.

The formation of prototrophic heterokaryons between auxotrophic mutants of *Streptomyces fradiae* was described by Braendle & Szybalski (1957). The frequency of heterokaryosis between compatible strains varied over a wide range, but was fairly reproducible for a particular parental combination during the limited period of study. Several months after these initial observations, some of the matings were repeated. In the intervening period, the auxotrophic mutants had been maintained in storage at 4°, undergoing several transfers and purification by single cell isolation. It was observed, in the case of the two mutants 6F4–1 (requiring methionine + isoleucine and 6F5–16 (requiring histidine + arginine), that the frequency of heterokaryosis on minimal agar had decreased from the earlier value of 100–200 colonies to less than one/inoculum of 10^8 conidia of each parent. On the other hand, re-isolation of the parental-type strains ('secondary parental isolates') from the conidia produced by these infrequent heterokaryons, and their subsequent mating, resulted in an increase in the frequency of heterokaryosis by a factor as high as 5000. This marked difference in mating ability suggested the possible existence of compatibility factors which controlled heterokaryotic mating.

Though the heterokaryotic phenomenon has received considerable attention (Dodge, 1942; Hansen, 1938; Beadle & Coonradt, 1944; Pontecorvo, 1946;

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Ishitani & Sakaguchi, 1956), only a few references have been made to the existence of preferential mating in heterokaryon formation (Papazian, 1950; Raper & San Antonio, 1954). The demonstration of heterokaryosis in many strains of Streptomyces has only recently been reported (Bradley & Lederberg, 1956; Szybalski & Braendle, 1956). The function of this phenomenon in the process of genetic recombination in the streptomycetes has been described for *Streptomyces coelicolor* (Sermonti & Spada-Sermonti, 1956) and for *S. fradiae* (Braendle & Szybalski, 1957). The following study was undertaken for the purpose of ascertaining the physiological and genetic basis of the striking difference in mating ability between auxotrophic mutants and their 'secondary parental isolates'.

METHODS

Stock cultures of mutants were maintained on Difco nutrient agar slopes enriched with 10 μg. required amino acid/ml. A defined minimal medium prepared with thoroughly washed agar (Braendle & Szybalski, 1957) was used in all mating experiments.

Auxotrophic mutants of *Streptomyces fradiae* 3535, *S. griseus* 3475, and *S. venezuelae* 3625 (from the culture collection of the Institute of Microbiology) were isolated after ultraviolet irradiation of the wild-type strains. The auxotrophic mutant, 14D1, of *S. coelicolor* was kindly sent to us by Dr G. Sermonti of the Istituto Superiore di Sanità, Rome.

Compatible strains were mated on minimal agar as described by Braendle & Szybalski (1957), and from the resulting heterokaryons 'secondary parental' strains were isolated as follows. Conidia were collected from the surface of the heterokaryotic colony with a wire loop and streaked on the surface of nutrient agar (NA) plates so as to obtain well-isolated colonies. The nutritional requirements of individual resulting colonies were determined and in all cases were identical with those of either parent. Representative colonies of both parental types were chosen at random and streaked on supplemented nutrient agar slants for future use. The 'secondary parental' strains thus isolated were designated by a capital letter after the code number of the primary parental strain; e.g. the two 'secondary parental' types isolated from a particular heterokaryotic colony composed of mutants 6F4–1 and 6F5–16 were designated 6F4–1A and 6F5–16A.

For the preparation of conidial suspensions in the mating experiments, conidia of suitable parental strains were harvested from slope or plate cultures, and suspended in 5 ml. saline, to which Triton WR-1339 (Winthrop-Stearns Inc.) was added in a final concentration of 0·05% (w/v). The suspensions were thoroughly agitated and filtered through Whatman No. 2 filter paper. The number of viable conidia was subsequently measured by plating appropriate dilutions on complete media and counting the number of colonies which appeared after 4 days of incubation at 28°. Heterokaryons were synthesized by spreading a mixture of 0·1 ml. of each of the appropriate parent conidial suspensions on the surface of a minimal agar plate. Heterokaryotic colonies were counted after incubation for 8 days at 28°.

The frequency of heterokaryosis was determined by calculating the ratio: average number of heterokaryotic colonies which appeared on the plates to number of conidia in the inoculum of the minority parent. This ratio, which will be referred to as the 'heterokaryotic frequency', remained reasonably constant for a particular parental combination when the inoculum varied between 10^7 and 10^9 conidia/plate.

To determine spontaneous reversion rates, each suspension of parental conidia was also plated separately on minimal agar plates, which were inspected for colony formation after incubation for 8 days.

A test for syntrophy was performed by streaking the mutant strains on minimal agar in close proximity to each other without permitting direct contact. Should cross-feeding occur under these conditions, growth would be expected to appear in regions where the streaks were most closely associated.

RESULTS

Heterokaryotic frequency of 'primary, secondary, and tertiary parental isolates'

Strains 6F4–1 (meth⁻, isoleu/leu⁻) and 6F5–16 (hist⁻, arg⁻), together with their 'secondary parental isolates' (6F4–1A, 6F4–1B, 6F5–16A, and 6F5–16B) bearing the respective parental deficiencies and derived from heterokaryons in the manner described in Methods, were mated in all possible combinations. The absence of growth on the control plates indicated that reverse mutation to prototrophy, and syntrophy between the parental auxotrophs, were negligible factors in these experiments. Table 1 summarizes the results, expressed as the frequency of heterokaryon formation. The recorded number of heterokaryotic colonies resulting from each mating is an average of four plates prepared simultaneously. A sharp increase in the frequency of heterokaryosis invariably resulted when the corresponding 'secondary parental isolates' were substituted for the parental auxotrophs. This held true even when the former originated from different heterokaryotic colonies of the same cross.

To determine whether strains with still higher frequencies of heterokaryosis could be developed by successive isolation of 'secondary' and 'tertiary' parental types from heterokaryons, conidial isolates were obtained from heterokaryons formed between strains 6F4–1A and 6F5–16A. These 'tertiary parental isolates', when mated, displayed the same order of heterokaryosis as did strains 6F4–1Aand 6F5–16A.

Germination of conidia and residual growth

It was conceivable that the observed increase in frequency of heterokaryosis might be the result of selection for conidia with higher germination capacity or greater residual mycelial development, thereby increasing the possibility of direct contact between the parental mycelia on the surface of the selective medium. To test this possibility, strains 6F4–1, 6F5–16, 6F4–1A, 6F5–16A, and 6F5–16B were plated separately and in compatible combination on a thin layer of minimal agar. Microscopic observation failed to show any noticeable differences in conidial germination and residual mycelial development between strain 6F4–1 and its high-frequency derivative 6F4–1A, when these strains were cultivated separately. Conidial germination for both strains was about 90%, with the length of the residual hyphal growth varying between 0·6 and 12μ at 24 hr. Growth terminated in 48 hr. with the development of small branched tangles of mycelium. Strains 6F5–16, 6F5–16A, and 6F5–16B appeared identical in their conidial germination and residual hyphal growth. Conidial germination for these strains amounted to 10–20% after incubation for 39 hr. The process of germination was 90% complete after 6 days of incubation, with hyphal length varying between 0·5 and 10μ; a small amount of branching was observed in strain 6F5–16B at this time. Further incubation of these cultures failed to promote growth beyond the 6-day limit.

Table 1. *Frequency of heterokaryosis between two auxotrophic mutants of* Streptomyces fradiae *and their 'secondary parental isolates'*

The 'secondary parental isolates' are indicated by the letter A or B following the symbol of the auxotrophic mutant 6F4–1 requiring methionine + isoleucine/leucine; and 6F5–16 requiring histidine + arginine.

Strains	Inoculum (no. of conidia/plate)	No. of heterokaryotic colonies/plate*	Heterokaryotic frequency × 10^8†
6F4–1	$4\cdot7 \times 10^7$	0·25	0·53
6F5–16	$8\cdot0 \times 10^7$		
6F4–1	$4\cdot7 \times 10^7$	4·2	28·0
6F5–16A	$1\cdot5 \times 10^7$		
6F4–1	$4\cdot7 \times 10^7$	3·5	7·5
6F5–16B	$4\cdot9 \times 10^7$		
6F4–1A	$3\cdot8 \times 10^8$	75·0	95·0
6F5–16	$8\cdot0 \times 10^7$		
6F4–1B	$2\cdot6 \times 10^9$	155	5·9
6F5–16	$1\cdot5 \times 10^{10}$		
6F4–1 A	$3\cdot8 \times 10^8$	521	3500
6F5–16A	$1\cdot5 \times 10^7$		
6F4–1A	$4\cdot6 \times 10^7$	2494	5400
6F5–16B	$1\cdot5 \times 10^8$		
6F4–1B	$2\cdot6 \times 10^9$	1655	63
6F5–16A	$3\cdot8 \times 10^9$		
6F4–1B	$1\cdot2 \times 10^7$	637	5300
6F5–16B	$4\cdot9 \times 10^7$		

* Average number of heterokaryotic colonies on four plates.

† Ratio of the number of heterokaryotic colonies to the number of conidia of the minority parent in the inoculum (cf. Methods).

When the strains were mixed in pairs, 6F4–1 and 6F5–16, 6F4–1A and 6F5–16A, 6F4–1A and 6F5–16B, germination and mycelial development appeared to be limited to that previously observed with strains 6F4–1 and 6F4–1A. In 39 hr. conidial germination varied from 70 to 80%, with hyphal development approximately 12μ in length and considerable branching apparent. The growth of the mixed culture of strains 6F4–1A and 6F5–16B was considerably advanced at this time, showing dense centres of mycelial growth which were ultimately to become heterokaryotic colonies. A few ungerminated conidia were apparent in matings between 6F4–1 and 6F5–16 after 6 days; however, growth was too extensive in the other mixed cultures for detailed observations. The number of heterokaryotic colonies on the plates observed microscopically varied from three colonies in the 6F4–1/6F5–16 mating to almost confluent colonial growth in the 6F4–1A/6F5–16A matings and 6F4–1A/6F5–16B matings, a result which correlated well with that obtained when the strains were previously mated, as shown in Table 1.

Stability of heterokaryotic compatibility

The stability of the condition of high frequency of heterokaryotic compatibility was tested under various conditions. HFM strains were stored for 6 months on nutrient agar slopes at 4°. Upon subsequent testing, they exhibited undiminished frequencies of heterokaryotic interaction.

The effect of serial transfer on the stability of the high-frequency of mating was also evaluated. The results obtained in the first experiment are summarized in Table 2, Expt. 1. The HFM strains were transferred from slope to slope every 5 days by mass mycelial inoculation. After every third transfer, crosses were made. Following nine serial transfers, the frequencies of heterokaryosis were markedly decreased. After 12 transfers, there was essentially no difference in mating ability between the 'secondary parental isolates' and the original auxotrophs.

Further experiments were initiated to ascertain whether the change from low- to high-mating frequency follows a single or multiple step pattern. If the first alternative were correct, it could then be postulated that different frequencies of mating reflect the presence of various proportions of high-frequency mating (HFM) 'mutants' in non-mating parental populations. Indirect isolations of the hypothetical HFM 'mutants' from the original parental population by replica plating (Lederberg & Lederberg, 1952) or other similar methods appeared, at first, to be the most promising approach to this question. That this method would be impractical, however, was indicated by an examination of the data presented in Table 1, which suggest that the hypothetical HFM 'mutant' must be very rare in the 6F4–1 or 6F5–16 parental populations because of the very low frequency of heterokaryon formation. An opposite approach was therefore adopted: indirect isolation of low-frequency mating (LFM) 'revertants' from an HFM strain which had undergone a few serial transfers. That such 'revertants' might be fairly common under these conditions was suggested by an earlier experiment, in which the frequency of heterokaryosis of the 'secondary parental isolates' was shown to decrease after several serial transfers. However, the experiment based on these premises produced unforeseen results, as represented in Table 2, Expt. 2. The frequency of heterokaryosis between the 'secondary parental isolates' 6F4–1A and 6F5–16A seemed to decrease up to the 8th transfer and then abruptly increased, reaching a very high value. Five single conidial isolates

Table 2. *Influence of serial transfers of high and low compatibility strains of* Streptomyces fradiae *on the frequency of heterokaryosis*

No. of transfers	Strains	Inoculum (no. of conidia/plate)	No. of heterokaryotic colonies/plate*	Heterokaryotic frequency $\times 10^{8}$*
		Experiment 1		
3	6F4–1 6F5–16	$1\cdot1\times10^{7}$ $5\cdot2\times10^{9}$	10·0	91·0
3	6F4–1A 6F5–16A	$9\cdot4\times10^{6}$ $1\cdot9\times10^{9}$	269	2800
6	6F4–1 6F5–16	$1\cdot4\times10^{6}$ $3\cdot7\times10^{7}$	7·5	53·0
6	6F4–1A 6F5–16A	$1\cdot1\times10^{7}$ $4\cdot1\times10^{7}$	400	3500
9	6F4–1 6F5–16	$3\cdot0\times10^{7}$ $5\cdot2\times10^{9}$	22·5	74·0
9	6F4–1A 6F5–16A	$2\cdot8\times10^{7}$ $1\cdot8\times10^{9}$	65·5	230
12	6F4–1 6F5–16	$6\cdot5\times10^{7}$ $1\cdot2\times10^{10}$	13·5	20·7
12	6F4–1A 6F5–16A	$1\cdot4\times10^{7}$ $2\cdot7\times10^{8}$	4·0	28·0
15	6F4–1 6F5–16	$8\cdot9\times10^{6}$ $9\cdot8\times10^{8}$	7·5	84·6
15	6F4–1A 6F5–16A	$1\cdot7\times10^{7}$ $4\cdot8\times10^{8}$	7·5	44·6
		Experiment 2		
3	6F4–1 6F5–16	$6\cdot1\times10^{7}$ $6\cdot3\times10^{7}$	13·5	22·0
3	6F4–1A 6F5–16A	$2\cdot8\times10^{7}$ $4\cdot3\times10^{7}$	383	1370
6	6F4–1 6F5–16	$5\cdot7\times10^{7}$ $1\cdot1\times10^{8}$	8·8	15·4
6	6F4–1A 6F5–16A	$1\cdot1\times10^{8}$ $2\cdot6\times10^{7}$	353	1300
7	6F4–1 6F5–16	$7\cdot7\times10^{7}$ $2\cdot3\times10^{7}$	3·0	13·0
7	6F4–1A 6F5–16A	$5\cdot5\times10^{7}$ $6\cdot3\times10^{7}$	387	703
8	6F4–1 6F5–16	$9\cdot6\times10^{7}$ $7\cdot9\times10^{7}$	13·8	17·4
8	6F4–1A 6F5–16A	$6\cdot2\times10^{7}$ $7\cdot9\times10^{7}$	321	518
12	6F4–1A 6F5–16A	$4\cdot2\times10^{7}$ $2\cdot1\times10^{7}$	357	1700
13	6F4–1A 6F5–16A	$9\cdot0\times10^{6}$ $9\cdot0\times10^{6}$	681	7570
14	6F4–1A 6F5–16A	$6\cdot0\times10^{7}$ $6\cdot1\times10^{7}$	>5000	>10000
15	6F4–1A 6F5–16A	$1\cdot1\times10^{8}$ $1\cdot6\times10^{8}$	>5000	>5000
21	6F4–1A 6F5–16A	$6\cdot9\times10^{6}$ $8\cdot7\times10^{7}$	>5000	>80000

* Cf. Table 1.

were selected from each HFM parent after the 7th and 12th transfers and crossed in all possible combinations (2 × 25 crosses). All of these crosses exhibited a very high heterokaryotic frequency, with confluent heterokaryotic growth on the majority of plates.

Interspecific mating

To study heterokaryotic compatibility between different strains and species of Streptomyces, isolates of *Streptomyces fradiae* capable of high-frequency heterokaryosis were mated with other auxotrophs of *S. fradiae* and with auxotrophs of other species of *Streptomyces*: 7A25 (*S. venezuelae* requiring isoleucine + valine), 4B19–1 (*S. griseus* requiring arginine + methionine), 14D1 (*S. coelicolor* requiring histidine + methionine), and 6F3–3 (*S. fradiae* requiring methionine + threonine). As shown in Table 3, no heterokaryons resulted from interspecific matings. When mutant 6F3–3 was mated with an HFM isolate of another auxotroph of *S. fradiae*, the result was a small but reproducible increase in the frequency of heterokaryosis over that observed for the two parental auxotrophs.

Table 3. *Frequency of heterokaryon formation in interspecific and intraspecific mating experiments*

Strains	Species	Inoculum (no. of conidia/plate)	No. of heterokaryotic colonies/plate*	Hetero-karyotic frequency*
6F4–1	*S. fradiae*	$4{\cdot}4\times10^{7}$	68·0	155
6F5–16	*S. fradiae*	$1{\cdot}5\times10^{8}$		
6F4–1	*S. fradiae*	$4{\cdot}4\times10^{7}$	470	1070
6F5–16A	*S. fradiae*	$3{\cdot}8\times10^{8}$		
7A25	*S. venezuelae*	$3{\cdot}0\times10^{9}$	0·0	—
6F5–16	*S. fradiae*	$1{\cdot}5\times10^{8}$		
7A25	*S. venezuelae*	$3{\cdot}0\times10^{9}$	0·0	—
6F5–16A	*S. fradiae*	$3{\cdot}8\times10^{8}$		
4B19–1	*S. griseus*	$6{\cdot}4\times10^{8}$	0·0	—
6F5–16	*S. fradiae*	$1{\cdot}5\times10^{8}$		
4B19–1	*S. griseus*	$6{\cdot}4\times10^{8}$	0·0	—
6F5–16A	*S. fradiae*	$3{\cdot}8\times10^{8}$		
14D1	*S. coelicolor*	$1{\cdot}5\times10^{8}$	0·0	—
6F5–16	*S. fradiae*	$1{\cdot}5\times10^{8}$		
14D1	*S. coelicolor*	$1{\cdot}5\times10^{8}$	0·0	—
6F5–16A	*S. fradiae*	$3{\cdot}8\times10^{8}$		
6F3–3	*S. fradiae*	$3{\cdot}1\times10^{9}$	13·7	0·9
6F5–16	*S. fradiae*	$1{\cdot}5\times10^{8}$		
6F3–3	*S. fradiae*	$3{\cdot}1\times10^{9}$	217	5·7
6F5–16A	*S. fradiae*	$3{\cdot}8\times10^{8}$		

* Cf. Table 1

DISCUSSION

The experimental data indicate that through the selection of parental-type conidia produced by heterokaryotic colonies, the frequency of heterokaryosis can be significantly increased over that characteristic for the original parents.

Two possible mechanisms for this phenomenon were evaluated: (1) greater residual mycelial growth of the secondary parental-type conidia on the selective medium, providing more opportunity for hyphal contact; (2) a greater frequency of anastomosis upon hyphal contact. Operation of the first mechanism appears unlikely, since comparative microscopic examination indicated that low- and high-frequency mating types were similar in frequency of conidial germination and in mycelial development, when grown separately or in mixed culture. At the present time, there are no obvious means of making direct observation or measurement of the frequency of hyphal fusion (anastomosis) in *Streptomyces fradiae* because of the rare occurrence of this process. The high frequency of cellular fusion in *S. scabies* reported by Gregory (1956) might present advantages from this aspect.

Some hint as to the nature of the inheritance of heterokaryotic compatibility can be obtained from the experiments in which the stability of the mating behaviour after serial transfer was studied. Table 2, Expt. 1, indicates that the frequency of heterokaryon formation remained high through several transfers, gradually decreasing after 9 transfers (corresponding to approximately 150 nuclear divisions and respective cytoplasmic growth), to the low value characteristic of the original mutants only after 12 transfers. If the number of nuclear divisions is considered equivalent to the number of cell divisions, as in the case of the Eubacteriales, loss of the ability to form heterokaryons takes place much too slowly to be caused by cytoplasmic dilution. On the other hand, this gradual decline in heterokaryotic frequency can be explained more readily in terms of a mutation and selection mechanism. If there were a slow rate of back mutation from high- to low-frequency mating type, provided the low-frequency mating type had a selective advantage, the composition of the high-frequency mating-type population would slowly change to that of the predominantly low-frequency mating-type parental strains.

Further confirmation of the mutation hypothesis is provided by Expt. 2 (Table 2). The fluctuation in the heterokaryotic frequency might be construed to represent successive population shifts, at first favouring LFM and later HFM mutants. This second shift of the whole population toward higher heterokaryotic frequency after the 12th transfer was accompanied by loss of the capacity of the strains to sporulate and a consequent decrease in the inoculum size with each successive transfer.

Attempts to derive strains demonstrating even higher frequencies of heterokaryotic colony formation through successive heterokaryotic mating and isolation of tertiary parental-type conidia failed to produce strains of higher mating frequency. On the other hand, higher heterokaryotic frequencies were observed after serial transfers in Expt. 2 (Table 2) and upon single conidium isolation during the same experiment. Further studies on the ability of HFM strains to form heterokaryons with strains of the same species but of different origin may prove helpful in the evaluation of heterokaryotic compatibility as a taxonomic criterion, since in no case were interspecific matings successful.

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REFERENCES

Beadle, G. W. & Coonradt, V. L. (1944). Heterokaryosis in *Neurospora crassa*. *Genetics*, **29**, 291.

Bradley, S. G. & Lederberg, J. (1956). Heterokaryosis in Streptomyces. *J. Bact.* **72**, 219.

Braendle, D. H. & Szybalski, W. (1957). Genetic interaction among streptomycetes: heterokaryosis and synkaryosis. *Proc. nat. Acad. Sci. Wash.* **43**, 947.

Dodge, B. O. (1942). Heterokaryotic vigor in Neurospora. *Bull. Torrey bot. Cl.* **69**, 75.

Gregory, K. F. (1956). Hyphal anastomosis and cytological aspects of *Streptomyces scabies*. *Canad. J. Microbiol.* **2**, 649.

Hansen, H. N. (1938). The dual phenomenon in imperfect fungi. *Mycologia*, **30**, 442.

Ishitani, C. & Sakaguchi, K. (1956). Hereditary variation and recombination in Koji-molds (*Aspergillus oryzae* and *Asp. sojae*). V. Heterocaryosis. *J. gen. Appl. Microbiol.* **2**, 345.

Lederberg, J. & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.

Papazian, H. P. (1950). Physiology of the incompatibility factors in *Schizophyllum commune*. *Bot. Gaz.* **112**, 143.

Pontecorvo, G. (1946). Genetic systems based on heterokaryosis. *Cold Spr. Harb. Symp. quant. Biol.* **11**, 193.

Raper, J. R. & San Antonio, J. P. (1954). Heterokaryotic mutagenesis in Hymenomycetes. I. Heterokaryosis in *Schizophyllum commune*. *Amer. J. Bot.* **41**, 69.

Sermonti, G. & Spada-Sermonti, I. (1956). Gene recombination in *Streptomyces coelicolor*. *J. gen. Microbiol.* **15**, 609.

Szybalski, W. & Braendle, D. H. (1956). Genetic recombination in Streptomyces. *Bact. Proc.* 48.

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CORRIGENDUM

D. H. Braendle, Beth Gardiner and W. Szybalski (1959)
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On page 442, footnote:

Delete †—Dr W. Szybalski's address remains unchanged: Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey, U.S.A.

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Studies on a Toxin-Inactivating Substance Excreted by *Penicillium cyaneo-fulvum*

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SUMMARY: The reaction between staphylococcal α-toxin and a toxin-inactivating substance (TIS) excreted by *Penicillium cyaneo-fulvum* was found to proceed optimally at about pH 7·8, and in the temperature range 36–44°. A modified assay method, based on these findings, was used to follow TIS excretion by the mould. Appearance of the active principle in the culture filtrate followed closely upon the period of active sugar utilization by the mould. TIS was excreted in quiescent cultures, but not in shake cultures. The presence of certain nutritional factors found in beef heart infusion appeared to be necessary for TIS excretion, since detoxifying activity could not be detected in filtrates from cultures of the mould grown in the absence of this nitrogen source. The marked correlation between detoxifying and protease activities of culture filtrates during growth of the mould under various conditions, as well as during purification of TIS, and its inactivation by heat and by DFP, suggested that both activities were manifestations of the same enzymic reaction.

The ability of certain moulds and bacteria to produce substances capable of inactivating bacterial toxins was first noted by Metchnikoff (1897). Other toxin-inactivating substances of microbial origin have since been reported (Ramon & Richou, 1945; Cavalli, 1947; Smolens, McAleer & McLaren, 1947; Del Vecchio, Del Vecchio, Napoli & Argenziano, 1948; Villa, 1950; Richou & Gerbeaux, 1952; Liu, 1954), although none has been characterized, nor have their modes of action been elucidated. Recently, Murray, Denton & Stevenson (1957) showed that filtrates from cultures of a strain of *Penicillium cyaneo-fulvum* were capable of detoxifying staphylococcal α- and β-haemolysins, streptolysins O and S, the toxins of *Corynebacterium diphtheriae* and *Haemophilus pertussis*, and clostridial toxins. These workers also reported that toxins which had been inactivated by this mould product retained their antigenic properties.

The results from a study of the reaction between staphylococcal α-toxin and the toxin-inactivating substance (TIS) excreted by *Penicillium cyaneo-fulvum* have been reported briefly by Rose & Martin (1958). The present paper gives a more detailed account of these studies, and of the development of an improved method for assaying TIS. Data are also presented which suggest that the detoxifying action of this mould product is due entirely to its proteolytic activity.

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METHODS

Organism. The strain of *Penicillium cyaneo-fulvum* used in this study was obtained from Dr B. B. Diena, the culture having been isolated originally by Denton (Murray *et al.* 1957). Stock cultures were stored in soil (Greene & Fred, 1934), while cultures used in routine studies were maintained on slopes of Sabouraud maltose agar ('Difco'). Spore suspensions for inoculation were obtained by flooding slope cultures with sterile water and scraping the mould surface with a sterile wire. Liquid media were inoculated with 1% (v/v) of spore suspension (turbidity reading 75–85 at 650 mμ measured with a Coleman Junior spectrophotometer). Both quiescent cultures and shake cultures (agitated at 200 rev./min. on a rotary shaker) were incubated at 28°.

Media. When growing *Penicillium cyaneo-fulvum* for the preparation of partially purified TIS, 500 ml. portions of glucose-beef heart infusion-peptone medium (Murray *et al.* 1957) were employed.

Excretion of TIS by the mould was studied in a basal medium (Table 1) containing one of the following nitrogen sources:

(*a*) Bacto beef heart infusion ('Difco'), 5% (w/v) and/or proteose peptone ('Difco') 1% (w/v).

(*b*) Vitamin-free casein acid hydrolysate (Wyeth Corporation), 2·5% (w/v).

(*c*) $(NH_4)_2SO_4$, 0·3% (w/v).

The final pH of each medium was 7·0. Media were dispensed in 50 ml. portions into 500 ml. conical flasks, and autoclaved at 15 lb./sq.in. for 5 min.

Table 1. *Basal medium for* Penicillium cyaneo-fulvum

	μg./l.		g./l.
Nicotinamide	1000	Glucose	10·0
Pyridoxine hydrochloride	1000	NaCl	2·5
Calcium-D-pantothenate	1000	KCl	0·2
p-Aminobenzoic acid	1000	$CaCl_2$	0·1
Thiamine	1000		
Riboflavin	400		
Biotin	2·2		

Production and partial purification of TIS. Cultures (500 ml.) in 2 l. Fernbach flasks were removed after 6–7 days quiescent incubation (when the pH had reached 8·5–8·7). Combined culture filtrates were adjusted to pH 7·0, transferred to a separating funnel and saturated with ammonium sulphate. The solid material which rose to the surface was recovered and redissolved in water. After exhaustive dialysis, the solution was freeze dried.

Purification of staphylococcal α-toxin. Staphylococcal α-toxin, in the form of culture filtrates of *Staphylococcus aureus* 24MA, supplied by Dr J. M. B. Corkhill, was purified as follows (Dr A. W. Jackson, 1957, private communication):

Filtrates were dialysed for 4 hr. against cold distilled water, and the pH adjusted to 4·6–4·8. Zinc acetate was added in the proportion of 1 ml. of a 1% (w/v) solution for each 10 ml. of filtrate, and the pH adjusted to 4·3. One half volume of cold 95% (v/v) ethanol was slowly added, the mixture being immersed in a dry ice + ethanol bath during the addition. Precipitated toxin was removed by centrifugation and was washed with a mixture of 16·5 ml. ethanol and 3·5 ml. physiological buffer at pH 7·8 (Jackson & Little, 1957). The precipitate was dissolved in a neutral 2% (w/v) ethylene diamine-tetra-acetate (EDTA), using 1 ml. of solution for each 10 ml. of original filtrate. The solution was finally dialysed against cold distilled water for 3 hr., using two or three changes of water. Toxin solution prepared by this procedure had approximately 30% of the activity of the original filtrates.

Assay of staphylococcal α-toxin. Toxin was assayed by determining its haemolytic activity in 0·5 ml. of a 1% (v/v) suspension of washed rabbit erythrocytes in physiological buffer, pH 7·8, after incubation at 37° for 1 hr. The potency of toxin solutions was expressed in Burnet Units, a unit being defined as the smallest quantity of toxin that will produce 50% haemolysis in 1 ml. of a 1% (v/v) suspension of erythrocytes, after incubation for 1 hr. at 37° (Roy, 1937). Toxin-inactivating activity was expressed in Burnet Unit equivalents, one TIS unit being defined as the amount of TIS required to inactivate one Burnet Unit of toxin.

Analytical methods. Protease activities were estimated by measuring the amount of tyrosine liberated during digestion of a 2% solution of sodium caseinate (Husain & McDonald, 1958). The reaction mixture consisted of 9·0 ml. of substrate (pH 7·8), to which was added 1·0 ml. of TIS solution (containing 2·0–5·0 mg./ml.) or 1·0 ml. of culture filtrate; control mixtures contained 1·0 ml. of water. At zero time and after 4 hr. incubation at 37°, 2·0 ml. samples of the reaction mixture were removed, mixed with an equal volume of 0·6 M-trichloroacetic acid, and filtered. The tyrosine contents of the filtrates were determined by the method of Anson (1938). Protease activity was expressed as the number of μg. of tyrosine liberated by 1 mg. of TIS or by 1 ml. of culture filtrate.

Sugar contents of media and of culture filtrates were determined by the method of Somogyi (1945), and protein contents by the ultraviolet absorption method of Warburg & Christian (1941), using the formula suggested by Kalckar (1947).

RESULTS

Assay of the toxin-inactivating substance

The method used by Murray *et al.* (1957) for determining TIS activity followed conventional serological techniques and involved adding an equal volume of filtrate or TIS solution to each tube of serially diluted crude toxin, and incubating for 1 hr. Washed rabbit erythrocytes were added to each of the tubes, which were then incubated at 37° for a further hour. The TIS activity of the filtrate or solution was calculated from the apparent decrease in toxin concentration. This method, however, was found to give inconsistent, and in some instances, completely erroneous results. Since a single reaction mixture containing excess substrate (toxin) and limiting amounts of TIS was clearly preferable to using a series of mixtures in which the toxin concentration varied continuously, it was decided that the detoxifying reaction could best be studied by reacting TIS with excess toxin, and then determining the residual toxin by serial dilution. Preliminary experiments had indicated that mould culture filtrates contained substances which interfered with the assay procedure. The effect of these substances on the haemolysis of rabbit erythrocytes would therefore be decreased substantially by serial dilution of a sample from a single reaction mixture. This is particularly important in view of the findings (Bernheimer, 1947; Jackson & Little, 1957) that haemolysis can be inhibited by certain sugars and organic acids. On the basis of possible interference by substances in the staphylococcal culture filtrate, it was also deemed advisable to employ purified toxin preparations. Finally, serial dilution of the samples taken from the single reaction mixture served as a convenient means of stopping the toxin + TIS reaction.

Factors affecting the inactivation of toxin by TIS

Haemolysis of rabbit erythrocytes by staphylococcal α-toxin occurred optimally at pH 7·8 (Fig. 1). This value was higher than that found by Jackson & Little (1957) who obtained maximum lysis at pH 6·8–7·0, using toxin from a different strain of *Staphylococcus aureus*. The effect of pH on the inactivation of toxin by TIS is also shown in Fig. 1. Maximum TIS activity was observed in the pH 7·6–8·0 region, with activity falling off rapidly above pH 8·0.

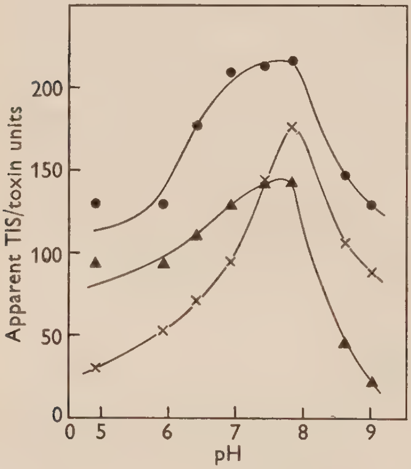

Fig. 1

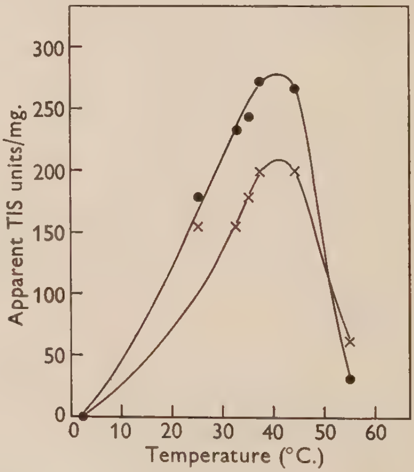

Fig. 2

Fig. 1. Effect of pH on lysis of rabbit erythrocytes by toxin (–×–×–), and on the inactivation of toxin by TIS (0·5 mg./ml.) after incubation for 1 hr. (–▲–▲–) and 2 hr. (–●–●–) at 37°. Toxin activity is in units/ml., and TIS activity in units/mg.

Fig. 2. Effect of temperature on the inactivation of toxin by TIS (0·5 mg./ml.) after incubation for 1 hr. (–×–×–) and 2 hr. (–●–●–) at pH 7·8. TIS activity is in units/mg.

Inactivation of toxin by TIS was also dependent upon temperature (Fig. 2), maximum activity being found in the range 36–44°. The apparently complete inhibition of the detoxifying reaction at 2–3° was of particular interest, in that it indicated that the reaction could be stopped by diluting a sample from a reaction mixture with cold buffer, a procedure which proved useful when following the course of the toxin + TIS reaction.

These results (Figs. 1 and 2) suggest that the inactivation of toxin is a relatively slow reaction, since considerable detoxification occurred during the second hour of incubation. The course of the toxin + TIS reaction was therefore examined in more detail. The results (Fig. 3) showed a linear relationship between TIS activity and time during the early part of the reaction, with the rate of reaction decreasing rapidly as detoxification neared completion.

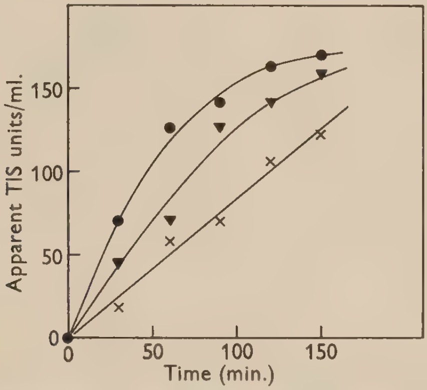

Fig. 3. Effect of incubation time on the inactivation of toxin by TIS at pH 7·8 and 37°. Concentrations of TIS used were 0·2 (–×–×–), 0·4 (–▼–▼–), and 0·6 (–●–●–) mg./ml. TIS activity is in units/ml.

Assay procedure. A method for assaying TIS activity was based on the observations reported above. To a portion of toxin (usually 0·5 ml.), adjusted to pH 7·8, was added an equal volume of TIS solution or culture filtrate diluted with physiological buffer at pH 7·8. The mixture was incubated for 2 hr. at 37°, along with control mixtures containing 0·5 ml. of water instead of toxin. The toxin contents of the control and of the reaction mixture were then determined as above. It was essential to adjust the TIS content of the reaction mixture such that not more than 75 % of the toxin in the mixture was inactivated, thereby ensuring that the rate of inactivation was linear during the 2 hr. incubation period. When assaying culture filtrates, it was necessary to dilute the filtrates with at least an equal volume of buffer, as they contained substances which interfered with the inactivation reaction. An incubation period of 2 hr. was considered suitable in that it enabled small amounts of activity to be measured.

Characterization of the toxin-inactivating substance

The results from the study of factors affecting inactivation of toxin by the mould product suggested a possible enzymic role for TIS. Purified preparations of TIS were therefore examined for enzyme activities that could explain their detoxifying action. This revealed that detoxifying activity in TIS preparations and in culture filtrates was always accompanied by marked protease activity. It was essential therefore to establish the relationship between toxin-inactivating and protease activity, and to ascertain the extent to which protease activity contributed to the detoxifying action of TIS preparations and of culture filtrates.

Figure 4 shows the course of TIS excretion by the mould growing in quiescent medium containing beef heart infusion and peptone, in relation to sugar utilization, and to changes in pH and protease content of the culture filtrate.

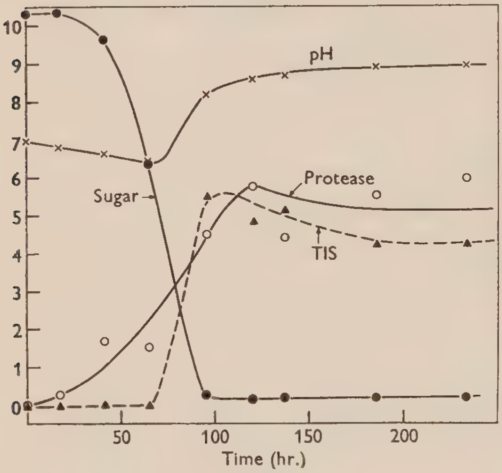

Fig. 4

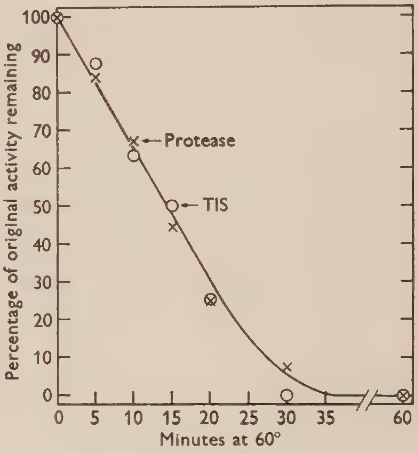

Fig. 5

Fig. 4. Excretion of TIS (–▲–▲–, units/ml.) and of protease (–○–○–, μg. tyrosine liberated/ml.) by the mould, in relation to sugar content (–●–●–, mg./ml.) and pH changes (–×–×–) in the culture filtrates.

Fig. 5. Effect of incubation at 60° on the toxin-inactivating (–○–○–, units/ml.) and protease (–×–×–, μg. tyrosine liberated/ml.) activities of a TIS preparation (2·0 mg./ml.).

Filtrates obtained during the first 60 hr. of incubation did not contain detectable amounts of TIS; but as sugar utilization neared completion, TIS was excreted. Protease activity was detectable in culture filtrates before TIS could be demonstrated, although both activities reached a maximum after 100–120 hr. incubation. Further incubation was accompanied by a slight decline in both activities.

When the mould was grown in a medium containing only beef heart infusion as a nitrogen source, the TIS and protease contents of culture filtrates did not reach a maximum until the cultures were 8 days old, corresponding to a delay of 24 hr. when compared with excretion in a beef-heart-infusion peptone medium. Filtrates from cultures grown in media containing peptone, casamino acids, or ammonium sulphate as a nitrogen source did not contain detectable amounts of TIS; slight protease activity was occasionally found in these filtrates, but this never attained the values that are associated with detoxifying activity (Table 2). Filtrates from shake cultures of the mould grown in media containing any of these nitrogen sources were also devoid of toxin-inactivating ability, although they frequently had slight protease activity (Table 2).

In order to examine further the correlation between toxin-inactivating and protease activities, the active principle (TIS) in filtrates from 6 to 7-day-old cultures of the mould, grown on a beef-heart-infusion peptone medium, was purified by copper sulphate precipitation (Diena, 1957), and ammonium sulphate fractionation. The following purification procedure was used: copper sulphate was added to the culture filtrate (350 ml.) to a concentration of 1 % (w/v), and the resulting precipitate removed by centrifugation and discarded. The precipitate obtained on adjusting the pH of the supernatant to 7·8 was also discarded. Saturation of the remaining solution with ammonium sulphate gave a precipitate which was dissolved in EDTA solution (M/1000) and reprecipitated at full saturation. This second precipitate (fraction I) was dissolved in EDTA solution, and the pH adjusted to 7·8. The precipitates obtained by adding ammonium sulphate to this solution to 40 % (fraction II) and 60 % (fraction III) saturation were then separated. Further fractions were obtained from an EDTA solution of fraction III by adding two volumes (fraction IV) and four volumes (fraction V) of saturated ammonium sulphate. Saturation of the remaining supernatant with ammonium sulphate gave the final fraction (fraction VI). All fractions were dissolved in M/1000 versene

Table 2. *Toxin-inactivating and protease activities of filtrates from cultures of* Penicillium cyaneo-fulvum *grown on media containing various nitrogen sources*

Nitrogen source	Quiescent (Q) or shaken (S) culture	Age of culture (hr.)	pH of culture filtrate	TIS content of culture filtrate (units/ml.)	Protease content of culture filtrate (μg. tyrosine liberated/ml.)
Beef heart infusion (5 %) + peptone (1 %)	Q	96	8·2	551	451
		186	8·9	438	552
	S	96	8·6	0	107
		186	9·0	0	152
Beef heart infusion (5 %)	Q	95	5·6	71	313
		161	8·8	355	490
	S	95	4·9	0	0
		161	8·9	0	2·0
Peptone (1 %)	Q	65	4·7	0	19·8
		161	8·7	0	49·5
	S	65	3·9	0	0
		161	8·7	0	0
Casein acid hydrolysate (1 %)	Q	91	4·8	0	0
		163	4·5	0	0
	S	91	4·7	0	0
		163	4·6	0	0
Ammonium sulphate (0·3 %)	Q	96	2·5	0	0
		186	2·5	0	0
	S	96	2·5	0	0
		186	2·4	0	0

solution, fraction I in 60 ml. (10 ml. retained for assay), fraction II in 10 ml., fraction III in 25 ml. (5 ml. retained for assay), and fractions IV, V and VI in 5 ml. Solutions were dialysed against distilled water, and adjusted to pH 7·8.

As shown in Table 3, this procedure for purifying TIS led to a seven-fold increase in specific activity (related to protein content). With each fraction, any increase or decrease in TIS activity was always accompanied by a corresponding change in protease activity.

Table 3. *TIS and protease activities of fractions from copper sulphate precipitation and ammonium sulphate fractionation of a mould culture filtrate*

Fraction	Protein content (mg./ml.)	TIS activity (units/mg. protein)	Protease activity (μg. tyrosine liberated/mg. protein)
Original culture filtrate	0·78	346	599
I	1·30	554	775
II	0·83	0	69
III	1·75	1223	1438
IV	1·80	2461	2876
V	4·35	494	111
VI	0·63	0	0

Table 4. *Effect of di*iso*propylfluorophosphate* (*DFP*) *on the toxin-inactivating and protease activities of a mould culture filtrate*

DFP, diluted in *iso*propanol, was added to 5·0 ml. portions of the culture filtrate (pH 7·0), and the total volume diluted to 6·0 ml. with phosphate buffer (pH 7·0). Samples were removed after 10 and 20 min. incubation at 37°, and immediately assayed for TIS and protease.

DFP concentration (M)	Period of incubation with DFP (min.)	TIS activity		Protease activity	
		Units/ml.	% activity remaining	μg. tyrosine liberated/ml. filtrate	% activity remaining
0	—	142	100	216	100
10^{-3}	10	0	0	1·4	1·2
	20	0	0	1·0	0·9
10^{-4}	10	71	50	90·8	42
	20	0	0	24	11
10^{-5}	10	142	100	202	93·5
	20	107	75	167	77·4

Incubation of TIS solutions (2·0 mg./ml.) in phosphate buffer (pH 7·8) at 60° for 60 min. led to the complete destruction of both toxin-inactivating and protease activities (Fig. 5). Shorter heat treatments brought about a quantitatively similar destruction of both activities (Fig. 5).

Treatment of proteolytic enzymes with various alkoxyphosphates, including di*iso*propylfluorophosphate (DFP) is also known to bring about an inhibition of enzyme activity (Balls & Jansen, 1952). When TIS solutions (2·0 mg./ml.) in phosphate buffer (pH 7·0) were treated with DFP at concentrations of

10^{-3} M or higher, there was a complete inhibition of detoxifying activity, and a near complete inhibition of protease activity. A small percentage of protease activity always remains after DFP inactivation (Balls & Jansen, 1952). Lower concentrations of DFP (10^{-4} M and 10^{-5} M) caused a partial, but nevertheless quantitatively similar inhibition of both activities (Table 4).

DISCUSSION

The findings presented in this paper substantially confirm the work of Murray *et al.* (1957), who demonstrated that culture filtrates of *Penicillium cyaneo-fulvum*, grown in a glucose-beef-heart-infusion peptone medium, contained a non-dialysable substance, capable of destroying the haemolytic activity of staphylococcal α-toxin. However, the observation that the detoxifying action was extremely rapid could not be substantiated. On the contrary, it was shown that, under the experimental conditions employed, the reaction was still proceeding after 2 hr. incubation. The modified assay for TIS gave consistent and reproducible results and, from both the theoretical and experimental aspects, appeared to be preferable to the method used by Murray *et al.* (1957).

Although the effects of pH, temperature and incubation time on the toxin-TIS reaction were examined primarily with the object of establishing the basis of an assay method, the curves showing the effects of pH and temperature suggest a catalytic role for the action of TIS on toxin. Moreover, the marked correlation between detoxifying and protease activities in all of the preparations studied, both during growth of the mould under various conditions and during purification of TIS, was extremely suggestive of a fundamental relationship between these two activities. The inability to demonstrate detoxifying activity in filtrates possessing low protease activity can probably be explained by a lack of sensitivity inherent in the TIS assay.

The possibility that both detoxifying and protease activities were manifested by the same agent became even more apparent from the closely similar effects of heat, and particularly of DFP on TIS preparations. Inactivation of proteolytic enzymes by DFP is thought to result from the introduction of *iso*propyl and phosphate groups on the active sites on the enzyme molecule, thereby preventing enzyme turnover (Jansen, Nutting, Jang & Balls, 1950). The fact that complete inhibition of protease activity in TIS preparations following DFP treatment was accompanied by the disappearance of detoxifying activity suggested that one or more of the active sites implicated in protein breakdown were also essential for toxin inactivation. This was further substantiated by the parallel effects of lower concentrations of DFP on the two activities. Protease inhibition by DFP is confined largely to endopeptidases, the inhibitor being ineffective against carboxypeptidase and many aminopeptidases (Neurath, 1957), which suggests that the enzyme excreted by *Penicillium cyaneo-fulvum* is an endopeptidase.

Direct evidence of a proteolytic degradation of toxin by detecting tyrosine liberation could not be established, due presumably to the extremely small amount of protein in the toxin preparations employed. Nevertheless, the data presented in this paper strongly suggest that the detoxifying activity in culture filtrates of *Penicillium cyaneo-fulvum* is wholly attributable to proteolytic degradation of the toxin protein. This does not preclude the possibility that several proteases are excreted by the mould, and that only certain of these are involved in toxin inactivation.

Inactivation of bacterial toxins by proteolytic enzymes has been noted by many workers (Walbum, 1929; Brandwijk & Tasman, 1937; Smythe & Harris, 1940; Parfentjev, Clapp & Waldschmidt, 1941). In one of the few detailed studies on this phenomenon, Parfentjev *et al.* (1941) observed that staphylococcal toxin that had been inactivated with pepsin retained its antigenic properties. The proteolytic activity of the detoxifying substance excreted by *Penicillium cyaneo-fulvum* could therefore explain the observed toxoiding action of TIS (Tanner, 1958).

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REFERENCES

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. gen. Physiol.* **22**, 78.

Balls, A. K. & Jansen, E. F. (1952). Stoichiometric inhibition of chymotrypsin. *Advanc. Enzymol.* **13**, 321.

Bernheimer, A. W. (1947). Comparative kinetics of haemolysis induced by bacterial and other haemolysins. *J. gen. Physiol.* **30**, 337.

Brandwijk, A. C. & Tasman, A. (1937). Action of trypsin on diphtheria toxin. *Lancet*, 1228.

Cavalli, L. L. (1947). Sostanze ad azione antidotica prodotte da miceti. *Boll. Ist. sieroter. Milano*, **26**, 149.

del Vecchio, G., del Vecchio, V., Napoli, A. & Argenziano, R. (1948). Richerche sul potere antidotico. V. Azione del complesso antidotico del liquido culturale di *P. notatum* sul potere antigene della tossina difterica. *Boll. Soc. ital. Biol. sper.* **24**, 186.

Diena, B. B. (1956). *A toxin-neutralising substance from* Penicillium cyaneo-fulvum: *Further studies.* Ph.D. Thesis, McGill University, Montreal.

Greene, H. C. & Fred, E. B. (1934). Maintenance of vigorous mould stock cultures. *Industr. Engng Chem.* (*Industr.*) **26**, 1297.

Husain, I. & McDonald, I. J. (1958). Characteristics of an extracellular proteinase from *Micrococcus Freudenreichii. Canad. J. Microbiol.* **4**, 237.

Jackson, A. W. & Little, R. M. (1957). Staphylococcal toxins. 1. Factors affecting the haemolytic activity of alphatoxin. *Canad. J. Microbiol.* **3**, 47.

Jansen, E. F., Nutting, M. D. F., Jang, R. & Balls, A. K. (1950). Mode of inhibition of chymotrypsin by diisopropyl fluorophosphate. II. Introduction of isopropyl and elimination of fluorine as hydrogen fluoride. *J. biol. Chem.* **185**, 209.

Kalckar, H. M. (1947). Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies on the enzymes of purine metabolism. *J. biol. Chem.* **167**, 461.

Liu, P. (1954). Inhibition of a staphylococcal haemolysin by a soluble substance produced by a non-haemolytic *Micrococcus* species. *J. Bact.* **68**, 718.

Metchnikoff, E. (1897). Ricerches sur l'influence de l'organism sur les toxines. *Ann. Inst. Pasteur*, **11**, 801.

Murray, E. D. G., Denton, D. G. & Stevenson, J. W. (1957). Toxin-inactivating substance and preparation. *British Patent* No. 787, 607. Dec. 11th.

Neurath, H. (1957). Some considerations of the multiple specificity of proteolytic enzymes. *Ann. N.Y. Acad. Sci.* **68**, 11.

Parfentjev, I. A., Clapp, F. L. & Waldschmidt, A. (1941). Staphylococcal toxoid prepared by peptic digestion. *J. Immunol.* **40**, 189.

Ramon, G. & Richou, R. (1945). De l'influence de la chaleur sur les propriétés antagonistes des filtrats de *B. subtilis* à l'égard des bacteries pathogènes et des toxines microbiennes. *C.R. Acad. Sci., Paris*, **221**, 213.

Richou, R. & Gerbeaux, C. (1952). Les complexes antagonistes. *Canad. J. comp. Med.* **16**, 311.

Rose, A. H. & Martin, S. M. (1958). Production and properties of a toxin-neutralising substance from *Penicillium cyaneo-fulvum*. *Bact. Proc.* 32.

Roy, T. E. (1937). The titration of alpha and beta haemolysins in staphylococcal toxin. *J. Immunol.* **33**, 437.

Smolens, J., McAleer, D. S. & McLaren, C. S. (1947). Antidotal properties of crude *Penicillium notatum* filtrate. *Proc. Soc. exp. Biol., N.Y.*, **65**, 172.

Smythe, C. V. & Harris, T. N. (1940). Some properties of a haemolysin produced by Group A β-haemolytic streptococci. *J. Immunol.* **38**, 283.

Somogyi, M. (1945). A new reagent for the determination of sugars. *J. biol. Chem.* **160**, 61.

Tanner, C. F. (1958). *The influence of a mold product on the antigenicity of staphylococcal toxin: Further studies.* Ph.D. Thesis, McGill University, Montreal.

Villa, A. M. (1950). Richerche su sostanze ad azione antidotica prodotte da miceti (Attivita dell *Aspergillus fumigatus*). *Mycopathologia (Amst.)*, **5**, 1.

Walbum, L. E. (1929). Toxin and antitoxin. In *Handbuch der Pathogen Mikroorganismen*, **2**, 537. Berlin: W. Kolle and A. V. Wasserman.

Warburg, O. & Christian, W. (1941). Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* **310**, 384.

(*Received* 30 *June* 1958)

Prokofieva-Belgovskaya, A. & Popova, L. (1959). *J. gen. Microbiol.* **20**, 462–472

The Influence of Phosphorus on the Development of *Streptomyces aureofaciens* and on its Ability to Produce Chlortetracycline

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SUMMARY: Parallel physiological and cytological investigations of a chlortetracycline-producing culture—*Streptomyces aureofaciens*, strain 536—indicate some intracellular causes of the dependence of chlortetracycline biosynthesis upon the inorganic phosphorus content of the nutrient medium.

Increase of inorganic phosphorus in the fermentation medium led to a formation of large nuclear elements containing much DNA (in corn steep medium) or ribonucleoproteid complexes in the shape of large volutin granules (corn steep liquor ash medium). As compared with the control, the nuclear substance/cytoplasm ratio in the hyphae is increased. The ability of mycelium overloaded with nuclear elements or volutin granules for chlortetracycline biosynthesis is markedly decreased. The influence of phosphorus on the microscopical structure of mycelium and biosynthesis of antibiotic depends on the composition of medium. The poor quality of certain batches of corn steep liquor used for the production of chlortetracycline is largely due to their high content of inorganic phosphorus.

Within recent years a number of reports have appeared concerning the significance of phosphorus for the production of antibiotics by various microorganisms (Biffi, Boretti, Di Marco & Penella, 1954; Boretti, Di Marco, Scotti & Zocchi, 1955; Di Marco, 1956; Shen *et al.* 1956; Prokofieva-Belgovskaya *et al.* 1957; Brinberg & Grabovskaya, 1958). However, the mechanism of its action upon the ability of a strain to synthesize antibiotics remains uncertain. The present investigation deals with the cytological aspect of this problem.

METHODS

Streptomyces aureofaciens, strain 536, obtained from the Selection Laboratory of this Institute was used in this investigation.

The inoculum was grown on a medium of the following composition: corn steep liquor (50 % solids), 4 g.; starch, 1·5 g.; water 100 ml.; pH before sterilization, 6·6–6·8.

Two media were used for the fermentation:

	(1) Standard medium	(2) Corn steep ash medium
Corn steep liquor (50 % solids)	1·0 g.	—
NH_4NO_3	0·5 g.	0·5 g.
NaCl	0·2 g.	0·2 g.
$CaCO_3$	0·4 g.	0·4 g.
Starch	2·0 g.	2·0 g.
Corn steep liquor ash	—	0·05 g.
Water	100 ml.	100 ml.
pH before sterilization	6·6–6·8	6·6–6·8

The fermentation of 125 ml. portions of medium was carried out in 750 ml. Erlenmeyer flasks, on a rotary shaker (200 rev./min.) at a temperature of 26–28° in the course of 72–80 hr. The fermentation medium was inoculated with 2–4 % (v/v) of the 26–27 hr. inoculum culture.

In the course of fermentation the following assays were made: chlortetracycline by the plate method with *Bacillus subtilis* as a test organism; carbohydrates and phosphorus. The cytological structure of mycelium was also studied.

For cytological analysis a drop of the fermentation broth was placed on a slide, dried and fixed with Carnoy fluid. The morphological structure of the mycelium was studied on preparations stained with methylene blue. The degree of the staining with this dye depends upon the isoelectric point of the protoplasm and serves at the same time as a good index for the RNA content of the protoplasm (Kelley, 1939). For this purpose pyronine may also be used.

To disclose nuclear elements and DNA a Feulgen reaction was used: the mycelium was hydrolysed in 1 N-HCl for 7 min. and stained with Schiff's reagent for 3 hr.

RESULTS

For the production of chlortetracycline a medium containing 1·0 % corn steep liquor was used in which phosphorus was present chiefly in the form of phytin and inorganic phosphorus. The total phosphorus contents in the corn steep liquor range from 3 to 5 % of the dry substance. Thus the amount of total phosphorus in the fermentation medium averages 200 mg./l. (~75 mg. free phosphorus/l.). In order to elucidate whether this amount of phosphorus ensures the optimal development of the culture and the biosynthesis of the antibiotic, experiments were performed with the introduction of additional amounts of phosphorus into two media. Phosphorus was introduced just before the inoculation in the form of potassium phosphate.

As the results of these experiments showed the addition of 0·5 g. phosphate/l. led to the same alterations in the fermentation process as those indicated by Di Marco (1956). A much smaller amount of the antibiotic was produced (Fig. 1). The pH value of the medium first fell considerably and then rose markedly. Carbohydrate was consumed more rapidly. More intense growth of the organism was also observed in the experimental flasks in the first hours of fermentation. The autolysis began at an earlier stage of culture development.

To trace phosphorus in the protoplasm of *Streptomyces aureofaciens* cytological and cytochemical studies of the culture were made in the course of its development on standard medium and on medium enriched with phosphorus.

The cytological changes of the culture during fermentation on corn steep medium are as follows.

During the first period of fermentation (from the inoculation to 20 hr. of growth) a mass of mycelia developed in the fermentation broth composed of septate hyphae with homogeneous highly basophilic protoplasm (Pl. 1, fig. 1). The loss of basophily of the protoplasm due to the action of ribonuclease and the low isoelectric point of the protoplasm (about pH 2·0–3·0; Hagedorn, 1955*a*; Prokofieva-Belgovskaya & Kapitonova, 1959) led to the conclusion that at this period its RNA content was high. This is confirmed by direct biochemical analysis by Biffi *et al.* (1954), Boretti *et al.* (1955), Guberniev, Ugoleva & Torbochkina (1956).

In the process of intensive growth connected with the synthesis of proteins the basophily of the protoplasm gradually decreased. The culture passed into its second stage of development characterized by the slow growth of the mycelial mass, which consisted of thin hyphae, and a low content of RNA in the protoplasm. It was possible at this stage to see numerous small nuclear elements in hyphae (Pl. 1, fig. 2). A fine nuclear reaction points to the presence of DNA in the nuclear substance (Pl. 2, fig. 6). This state of the protoplasm was continued till 72 hr. of growth and was characteristic of the period of intensive chlortetracycline production.

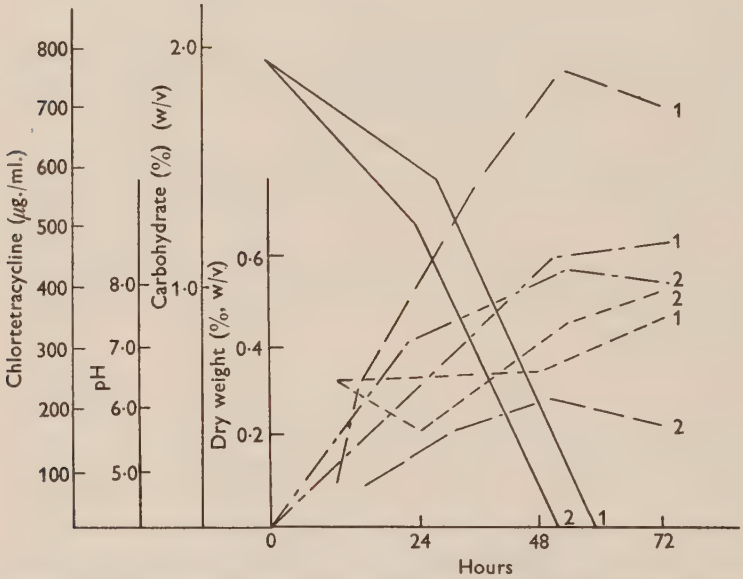


Fig. 1. Influence of inorganic phosphorus on the metabolism of *Streptomyces aureofaciens* and the chlortetracycline production (corn steep medium). 1, Standard corn steep medium; 2, standard corn steep medium + 0·05 % KH_2PO_4. ——, Carbohydrate; - - - - -, pH; — — — —, mycelium (dry weight); — — —, chlortetracyclin.

In some fermentations after 30–36 hr. of growth there developed among the thin hyphae single thickened and fragmented ones, containing intensely basophilic protoplasm and large nuclear elements rich in DNA (Pl. 1, fig. 2). But in highly productive fermentations the number of such hyphae was very small or they were completely lacking. When 0·5 g. phosphate/l. was added to the corn steep medium the development of the culture was markedly altered. After 24–30 hr. of growth a mycelial mass developed, consisting mostly of thickened fragmented, septate or catenulate hyphae with a highly basophilic protoplasm (Pl. 1, fig. 3). The intense staining of the protoplasm with pyronine pointed to a high RNA content. Nuclear staining showed that each fragment of these hyphae contained large nuclear elements occupying a considerable part of the fragment's volume (Pl. 2, fig. 6). The intensity of the nuclear reaction led to the conclusion that the DNA content in the nuclear elements was extremely high.

The chief mass of mycelium preserved such a structure till 40–48 hr. of growth. From 36 hr. more thin secondary hyphae developed in the culture which by that time already contained less nutritive substances, including inorganic phosphorus. By 48 hr. the growth of mycelium reached its maximum, the autolysis starting afterwards. Antibiotic content was extremely low.

To determine the ratio of the mass of nuclear elements to that of cytoplasm in the hyphae, the necessary measurements were made on preparations stained after Feulgen. Thin hyphae grown in standard medium (Pl. 2, fig. 6) and the enlarged fragmented hyphae which developed in the medium enriched with phosphorus (Pl. 2, figs. 7, 8) were investigated. The diameters of the nuclear elements were assumed to be equal to those of the hyphae. Such assumption does not involve a significant error and was in reasonable agreement with the findings of the present investigation as well as with the photomicrographs of other authors (Hagedorn, 1955*b*; Peshkov *et al.* 1952; Prokofieva-Belgovskaya & Demianovskaya, 1957; Di Marco, 1956). The cell wall was not stained by the technique used.

Table 1. *The ratio of the mass of nuclear elements to that of cytoplasm in the hyphae of mycelium grown on the standard medium, and of mycelium, developed in the medium enriched with phosphates*

	Total length of hyphae measured (μ)	No. of nuclear elements	Average diameter of the nuclear elements (μ)	Range of variation (μ)	Total volume of nuclear elements (μ^3)	Total volume of cytoplasm (μ^3)	Ratio of volume of nuclear elements to cytoplasm
Corn steep medium	188	114	0·29	0·1–0·4	2·2	11·85	0·18
Corn steep medium + 0·05 % phosphates	206	143	0·41	0·3–0·6	6·9	20·80	0·33

Table 1 shows that the mass of cytoplasm in the mycelium enriched with phosphorus was relatively decreased, while that of nuclear elements is increased which leads to a strong shift in the ratio of the fundamental cell components: nucleus/cytoplasm, in favour of the former; its relative quantitative increase is 1·8 times. The content of DNA determined microspectrophotometrically in such nucleoids increased approximately by a factor of 2·5 (Prokofieva-Belgovskaya & Maslov, to be published).

This type of development became more marked when inorganic phosphorus was introduced into the medium in the presence of antifoam agents. Sunflower oil was used as an antifoam agent. It was introduced into the medium at 0·5 % (v/v) at inoculation. In the presence of 0·05 % phosphate in this case the hyphae of the entire mass of mycelia were highly basophilic up to 40 hr. of growth. After 12 hr. growth they became fragmented, containing large nuclear elements rich in DNA (Pl. 3, fig. 9). Carbohydrates were rapidly consumed, the medium became more acid and less mycelial mass was accumulated by 72 hr. of fermentation (Table 2). The mycelium in the experimental flasks was only slightly pigmented, biosynthesis of the antibiotic was almost completely suppressed.

Table 2. *The influence of inorganic phosphorus on chlortetracycline synthesis and metabolism culture in a fermentation medium containing corn steep medium* + 0·5 % (*v*/*v*) *sunflower oil*

	Chlortetracycline		pH					Carbohydrate contents g./100 ml.		Mycelium (dry weight), g./100 ml., 72 hr.
KH_2PO_4 (g./100 ml.)	mg./l.	Inhib. (%)	Initial	6 hr.	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	
0·05	26	97·6	6·6	6·5	6·0	6·6	7·8	1·35	0·15	0·74
Control	1076	—	6·6	6·6	6·6	6·4	6·8	1·76	0·75	0·98

Table 3. *The influence of various concentrations of inorganic phosphorus on the production of chlortetracycline*

	Corn steep medium. Chlortetracycline		Corn steep medium + 0·5 % sunflower oil. Chlortetracycline	
KH_2PO_4 (g./100 ml.)	mg./l.	Inhib. (%)	mg./l.	Inhib. (%)
0·01	856	13	619	24
0·02	739	25	326	60
0·03	512	48	212	74
0·04	—	—	122	85
Control	985	—	814	—

Further investigations showed that even the introduction of trifling quantities of potassium phosphate into the medium (0·1 g./l.) decreased the chlortetracycline biosynthesis by 13 % when fermentation took place in corn steep medium and by 24 % in corn steep medium with antifoam agent (Table 3).

Both the total and the inorganic phosphorus content changed in the course of the fermentation. Phosphorus was taken up by the organism chiefly during the first day of growth. When grown in the presence of large amounts of phosphorus mycelium contains more phosphorus than on ordinary medium (Fig. 2).

Studies were carried out on the comparison of the corn steep liquors containing different amounts of inorganic phosphorus (Table 4).

In two of the corn steep liquors tested (25 and K-78), inorganic phosphorus was present in much larger quantities than in the rest. Experiments show that the smallest amount of antibiotic was produced on medium containing corn steep liquor with a comparatively large amount of inorganic phosphorus (25 and K-78). The utilization of corn steep liquors containing more than 0·9 % inorganic phosphorus in the fermentation medium led, as a rule, to the same

Table 4. *The influence of different batches of corn steep liquor on biosynthesis of chlortetracycline*

Experiment	Corn steep liquor batch no.	Phosphorus content in corn steep liquor (%)		Chlortetracycline	
		Total	Inorganic	mg./l.	% of control
1	25	3·9	2·3	130	8
	161*	4·3	0·95	1690	100
2	78	2·1	0·7	610	80
	80	3·9	0·9	620	80
	71	4·6	0·6	750	97
	161*	4·3	0·95	770	100
	K-78	3·6	2·2	60	10

* No. 161 is the control in this experiment, for it was used in all preceding experiments.

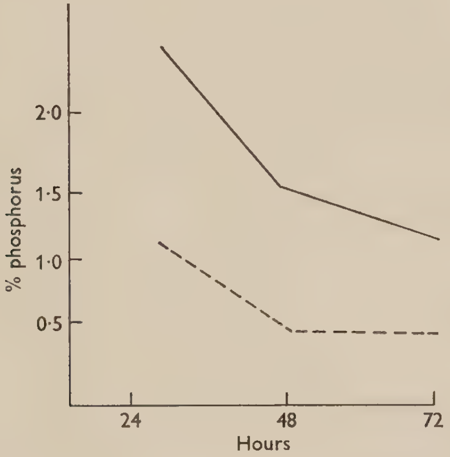


Fig. 2. Contents of phosphorus in mycelium grown in: standard corn steep medium, - - - - -; standard corn steep medium + 0·05 % KH_2PO_4, ——.

changes in the structure and development of the mycelium as those that occurred on medium to which 0·05 % phosphates were added (Pl. 3, figs. 10, 11). Markedly basophilic septate hyphae with large nuclear elements containing a considerable amount of DNA developed in the culture (Pl. 2, fig. 8). Chlortetracycline production by such mycelia is extremely weak (60–150 mg./l.).

The mycelium which developed in a medium containing corn steep liquor ash differed at all stages of growth from that found in standard medium by its thinner hyphae, smaller amount of RNA in the protoplasm and smaller nuclear elements with a less distinct nuclear reaction (Pl. 1, fig. 4).

When this medium was enriched with phosphates (0·1–0·5 g./l.) the actinomycete reacted in a different way than on standard medium. Addition of only 0·1 g. phosphate/l. sufficed to induce after 36 hr. of growth the appearance in the weakly basophilic protoplasm of volutin granules, which after 48 hr. form large blocks in the protoplasm (Pl. 1, fig. 5). Chlortetracycline synthesis was also markedly decreased (Table 5).

Table 5. *The influence of inorganic phosphorus on the biosynthesis of chlortetracycline on corn steep liquor ash medium*

KH_2PO_4 (g./100 ml.)	Chlortetracyclin mg./l.	Chlortetracyclin Inhib. (%)
0·01	180	56
0·02	30	93
0·05	0	100
Control	410	—

DISCUSSION

Biochemical data show (Demyanovskaya & Belozersky, 1954) that 72–76 % of actinomycete mycelium consists of nucleoproteins. The nucleic acid content of these varies from 11 to 17 %.

The ability of *Streptomyces aureofaciens* to produce chlortetracycline is closely connected with a definite state of the protoplasm characteristic for the second phase of its development. One of the major indices of this is the state of the nucleoproteins in the cytoplasm and the nuclear elements (Scotti & Zocchi, 1955; Prokofieva-Belgovskaya, Pestereva & Rudaya, 1956; Prokofieva-Belgovskaya & Orlova, 1956). A comparison of the physiological, cytochemical and biochemical data (Carvajal, 1947; Bringmann, 1951; Peshkov *et al.* 1952; Prokofieva-Belgovskaya *et al.* 1956; Prokofieva-Belgovskaya & Demianovskaya, 1957) led to the conclusion that the major intracellular factor which determines the changes in metabolism and the transition from the first phase of development (intensive growth) to the second phase (retarded growth and intensive production of the antibiotic) is the decrease in the RNA content of the protoplasm. The maximum amount of RNA in the protoplasm (according to cytochemical data and biochemical analysis) is observed during the period of 10–12 hr. growth, or 20–30 hr. before the maximum accumulation of mycelium. Hence, the actinomycete cell at first accumulates a maximum amount of nucleic acid and then utilizes it during its later growth. These findings correspond to those obtained with other organisms (Vendrely, Tulasne & Minck, 1948).

A number of investigations (Boretti *et al.* 1955; Biffi *et al.* 1954; Di Marco, 1956; Shen *et al.* 1956; Brinberg & Grabovskaya, 1958; Popova, to be published), like the present work, have established that the rise in the inorganic phosphorus contents of the fermentation medium leads to an increase in the amount of phosphorus-containing compounds in the mycelium. The physiological properties of such mycelia are markedly altered: the growth of the culture is accelerated, so is its consumption of carbohydrates, the synthesis of the phosphorus-containing components of the protoplasm—RNA and DNA—is increased, thicker hyphae develop which remain basophilic for a long time; the ability these hyphae have for the biosynthesis of the antibiotic is decreased.

A number of investigators (Shen *et al.* 1956; Di Marco, 1956) connect the inhibition of chlortetracycline synthesis under these conditions with the change in carbohydrate metabolism.

The development of *Streptomyces aureofaciens* culture may proceed in two different directions depending upon the inorganic phosphorus contents of the corn steep medium. Each of these is characterized by cytological peculiarities of the growth of the culture and its development, physiological properties and its ability to synthesize antibiotic. These different lines of development are determined by the relation between the structural elements of the cell: the cytoplasm and the nuclear elements.

Type I. The accumulation of nuclear substance in the hyphae is relatively low, its DNA content is small (a faint nuclear reaction), the growth of mycelium is connected chiefly with the accumulation of cytoplasm in the thin hyphae, the RNA content of which drops progressively. The ability for synthesis of the antibiotic is high (standard corn steep medium).

Type II. Nuclear substance accumulates intensively in the protoplasm in the form of large nuclear elements rich in DNA. The increase in nuclear substance by means of the reproduction of nuclear elements and their increase in size is accompanied by the inhibition of the growth of the cytoplasmic body of the cell. The thick hyphae are fragmented and show a tendency towards cell-formation. The ability for synthesizing antibiotic is markedly decreased (on corn steep medium enriched with inorganic phosphorus).

A comparison of our data with those of Di Marco (1956) and Shen *et al.* (1956) shows that there probably exists a strong interdependence between the synthesis of nucleic acids, the pathways of carbohydrate metabolism, and the capacity of *Streptomyces aureofaciens* culture to synthesize chlortetracycline. This conclusion is supported by the fact that when the conditions of aeration become worse and modify the pathways of the carbohydrate metabolism in accordance with the Embden-Meyerhof scheme, the microscopical structure and physiological properties of mycelium change. This change is similar to those observed after the enrichment of the medium with phosphorus (Dr Zolotnitsky, unpublished).

Of great importance also is the fact that fermentation of cultures producing streptomycin, terramycin and nistatin on media enriched with phosphorus leads to changes in their microscopical structure and physiological properties which are similar to the changes occurring in *Streptomyces aureofaciens* (Prokofieva-Belgovskaya *et al.* 1957).

Cytological studies of multicellular organisms have long ago established the general fact that when the nucleus passes into the mitotic state conditioned by the reduplication of chromosomes and the increase in their DNA contents, the cell comes out of its 'metabolic' or 'working' state (Berill & Huskins, 1936; Peter, 1940; Zalkind, 1952; and others); its specific functions are now completely inhibited (starch formation, the formation of glycogen, etc.). It is probable that in a medium containing a large amount of phosphorus the actinomycete cell behaves in the same way. Its metabolism under these conditions proceeds in the direction of the synthesis of nucleic acids (RNA and DNA) and the reduplication of nuclear elements, but not in the direction of the specific antibiotic synthesis connected with the growth of the cytoplasmic body of the cell.

It is possible that the increase of the size of nucleoids and of their DNA content is determined by the reproduction of the elementary nuclear structures of which the nucleoid is composed, this process being analogous to the polytenization of chromosomes in plants and animals.

The enrichment of actinomycete protoplasm with phosphorus in a medium with corn steep liquor ash stimulates not the reproduction of large nuclear elements with a high DNA content, but the synthesis and accumulation in the protoplasm of complex combinations of RNA and metaphosphates. Such are, evidently, the volutin granules described by Belozersky (1945) and Wiame (1945). This disturbance of nucleic acid metabolism in the protoplasm also leads to the decrease in capacity to synthesize chlortetracycline.

The data showing that the rise in inorganic phosphorus in the medium influences first of all the nucleic acid metabolism agree well with the fact that the chief mass of phosphorus in the medium is used by the organism during the first days of its growth, i.e. when the RNA synthesis is at its highest (Brinberg & Grabovskaya, 1958).

The action of inorganic phosphorus differs from that of its organic compounds (L. Popova, to be published). The introduction of phytin into the medium did not exert any noticeable effect on the cytology of the culture and its development or on its physiological properties, including the ability for the biosynthesis of the antibiotic.

The experiments with batches of corn steep liquor lead one to believe that the difference in productivity of separate batches of corn steep liquor depends upon difference in their inorganic phosphorus content. Batches of corn steep liquor with a large amount of inorganic phosphorus stimulate the development of mycelium overloaded with nuclear elements with high contents of DNA and possessing a low ability for chlortetracycline synthesis.

REFERENCES

Belozersky, A. N. (1945). On the chemical nature of volutin. *Microbiology, Moscow*, **14**, 1, 29.

Berill, N. & Huskins, C. (1936). The 'resting' nucleus. *Amer. Nat.* **70**, 257.

Biffi, G., Boretti, G., Di Marco, A. & Penella, P. (1954). Metabolic behaviour and chlortetracyclin production by *Streptomyces aureofaciens* in liquid culture. *Appl. Microbiol.* **2**, 288.

Boretti, G., Di Marco, A., Scotti, T. & Zocchi, P. (1955). Variazioni morphologiche e biochimiche dello *Streptomyces aureofaciens* in relazione alla produzione di chlorotetracyclina. *G. Microbiol.* **1**, 97.

Brinberg, S. L. & Grabovskaya, O. B. (1958). The significance of phosphorus for the biosynthesis of streptomycin. *Microbiology, Moscow*, **27**, 407.

Bringmann, G. (1951). Elektromikroskopische und Lichtmikroskopische Studien zur Morphologie und Entwicklung von *Streptomyces griseus* Krainsky. *Zbl. Bakt.* **157**, 349.

Carvajal, F. (1947). The production of spores in submerged cultures by some *Streptomyces*. *Mycologia*, **39**, 425.

Demianovskaya, N. S. & Belozersky, A. N. (1954). Desoxyribonucleic acid of *Actinomyces globisporus streptomycini* Kras. in the process of its development. *Biochem. Leningrad*, **19**, 688.

Guberniev, M. A., Ugoleva, N. A. & Torbochkina, L. M. (1956). Nucleic acids and phosphorus compounds in the mycelium of *Actinomyces aureofaciens* at different stages of development. *Antibiotiki, Moscow*, **3**, 8.

Hagedorn, H. (1955*a*). Untersuchungen über den isoelektrischen Punkt bei Actinomyceten. *Protoplasma*, **45**, no. 1, 115.

Hagedorn, H. (1955*b*). Beiträge zur Cytologie und Morphologie der Actinomyceten. *Z. Bakt.* (II. Abt.), **108**, 353.

Kelley, E. (1939). Reactions of dyes with cell substances. *J. biol. Chem.* **127**, 55.

Marco, A. Di (1956). Metabolism of *Streptomyces aureofaciens* and biosynthesis of chlortetracyclin. *G. microbiol.* **2**, 285.

Peshkov, M. A., Rautenstein, J. I., Sorokina, M. M., Cherednichenko, A. F. & Sharkova, A. S. (1952). Cytological changes of the *Actinomyces globisporus streptomycini* mycelium under the influence of actinophage. *Microbiology, Moscow*, **21**, 665.

Peter, K. (1940). Die indirekte Teilung der Zelle in ihren Beziehungen zu Tätigkeit, Differenzierung und Wachstum. *Z. Zellforsch.* **30**, 721.

Prokofieva-Belgovskaya, A. A. & Demianovskaya, N. S. (1957). Cytological and biochemical changes in *Actinomyces globisporus streptomycini* Kras. in the process of antibiotic production in submerged culture. *Microbiology, Moscow*, **26**, 22.

Prokofieva-Belgovskaya, A. A. & Kapitonova, O. N. (1959). Isoelectric point of cytoplasm and nuclear elements of *Actinomyces streptomycini* Kras. *Bull. Acad. Sci., U.S.S.R.* (in the Press).

Prokofieva-Belgovskaya, A. A. & Orlova, N. V. (1956). The peculiarities of growth and development of actinomycetes producing streptomycin, biomycin, and terramycin in submerged cultures. *Bull. Acad. Sci., U.S.S.R.* **5**, 59.

Prokofieva-Belgovskaya, A. A., Pestereva, G. D. & Rudaya, S. M. (1956). Peculiarities of growth and development of *Actinomyces rimosus* under the conditions of bottom fermentation. *Microbiology, Moscow*, **25**, 668.

Prokofieva-Belgovskaya, A. A., Popova, L. A., Orlova, N. V., Severin, V. A., Gorskaya, S. V. & Kapitonova, O. N. (1957). Dependence of the antibiotic activity of actinomycetes on the peculiarities of their growth and development. *Abstracts of reports presented at the Second All-Union Conference on Antibiotics* (Russian), 52.

Scotti, T. & Zocchi, P. (1955). Studio della struttura del micelio di Streptomyces aureofaciens. *G. microbiol.* **1**, 35.

Shen, S. C., Soohg, H. Y., Chen, J. P., Hung, M. M. & Yin, H. C. (1956). III. The effect of phosphate on the utilization of carbohydrates by *Streptomyces aureofaciens* and the production of aureomycin. *Acta Exp. Biol. Sinica*, **5**, 2.

Vendrely, R., Tulasne, R. & Minck, R. (1948). Premières observations sur les modifications chimiques qu'apporte la penicilline chez le Staphylocoque. *C.R. Soc. Biol., Paris*, **142**, 239.

Wiame, J. (1954). Presence of a polyphosphoric acid nucleic derivative in yeast. *C.R. Soc. Biol., Paris*, **140**, 897.

Zalkind, S. J. (1952). Mitosis and the functional activity of the cell. *Advanc. Modern Biol.* **33**, 431 (Russian).

EXPLANATION OF PLATES

The mycelium of *Streptomyces aureofaciens* were fixed with Carnoy fluid and stained with methylene blue (1:1000) or after Feulgen. Photomicrographs were made with a Zeiss microscope, ob. 90, N.A. 1·3, ocular ×15.

PLATE 1

magnification ×2500.

Fig. 1. Mycelium after 18 hr. growth on a standard corn steep medium. Hyphae with a homogeneous basophilic protoplasm. Methylene blue.

Fig. 2. Mycelium after 40 hr. growth on a standard corn steep medium. Decrease of the protoplasm basophily and the differentiation in it of fine nuclear elements. Methylene blue.

Fig. 3. Mycelium after 40 hr. growth on a corn steep medium + 0·05 % phosphates. Hyphae are fragmented, the protoplasm highly basophilic. Methylene blue.

Fig. 4. Mycelium after 48 hr. growth on a corn steep ash medium. Methylene blue.

Fig. 5. Mycelium after 48 hr. growth on a corn steep ash medium + 0·01 % phosphates. The protoplasm contains large volutin granules. Methylene blue.

PLATE 2

magnification ×1964.

Fig. 6. Mycelium after 40 hr. growth on a standard corn steep medium. A weak nuclear reaction of the nuclear elements. Feulgen.

Fig. 7. The nuclear reaction of mycelium after 40 hr. growth on a corn steep medium + 0·05 % phosphates. The fragmented protoplasm contains large nuclear elements with high content of DNA. Feulgen.

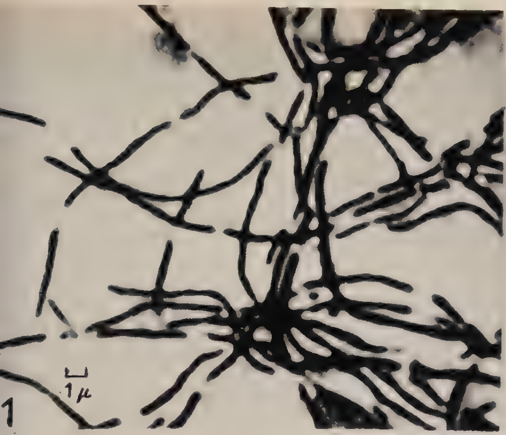
Fig. 8. Mycelium after 40 hr. growth on a standard medium with the corn steep liquor no. 25. A pronounced nucleal reaction of large nuclear elements. Feulgen.

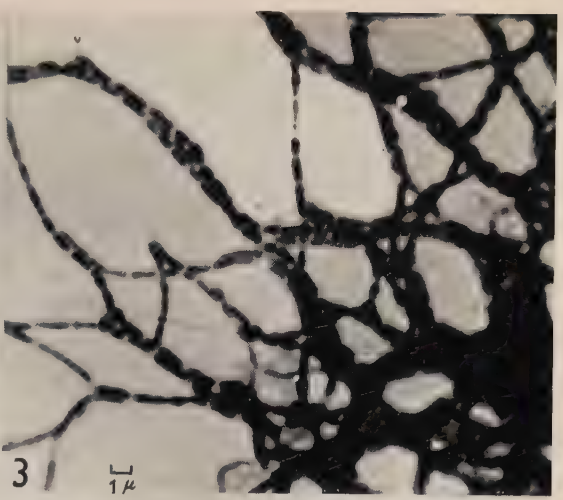
Fig. 9. Mycelium after 36 hr. growth on a corn steep medium + 0·05 % phosphates in the presence of antifoam agent. Methylene blue.

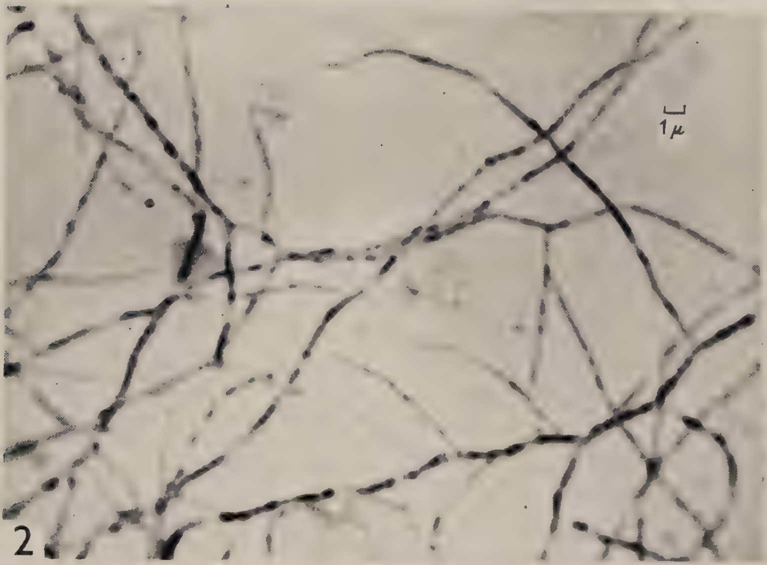
Fig. 10. Mycelium after 40 hr. growth on a standard medium with a corn steep liquor no. 161 (0·95 % of inorganic phosphorus). Methylene blue.

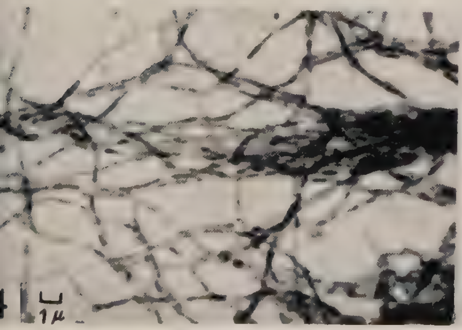
Fig. 11. Mycelium after 40 hr. growth on a standard medium with corn steep liquor no. 25 (2·2 % of inorganic phosphorus). Methylene blue.

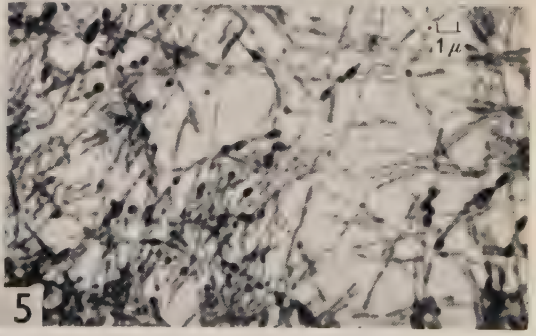
(*Received 7 August* 1958)





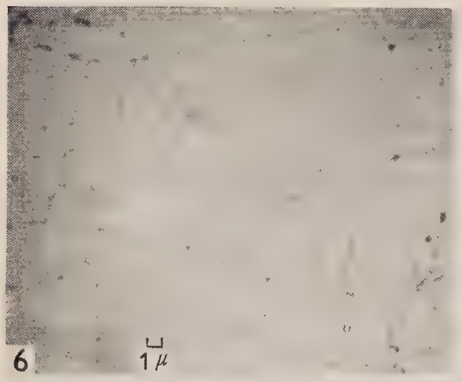


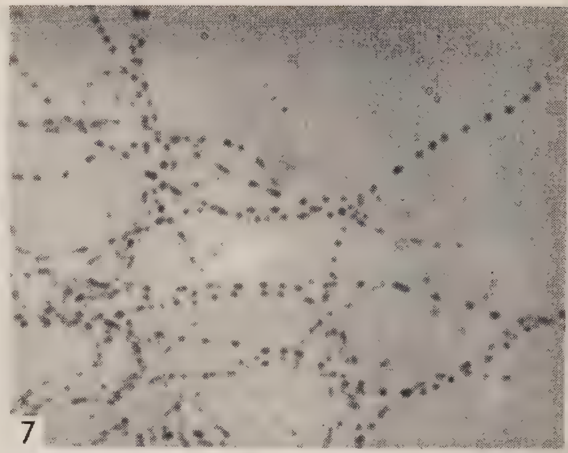


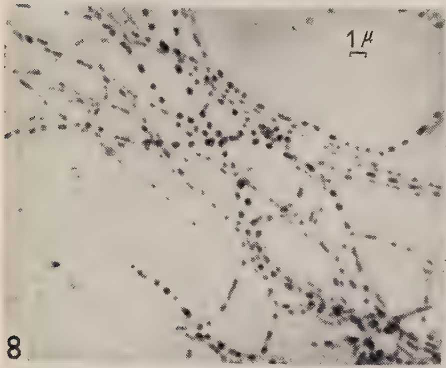


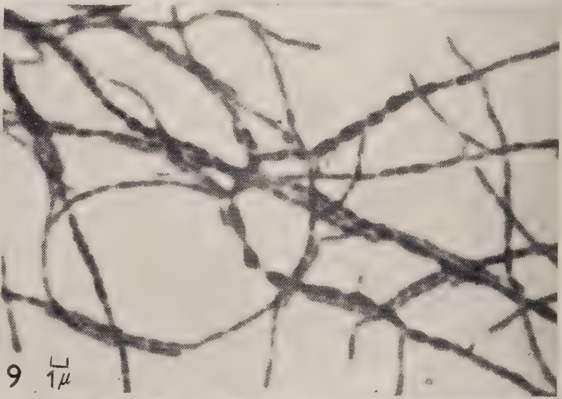
A. PROKOFIEVA-BELGOVSKAYA & L. POPOVA—CHLORTETRACYCLINE PRODUCTION BY *S. AUREOFACIENS*. PLATE 1

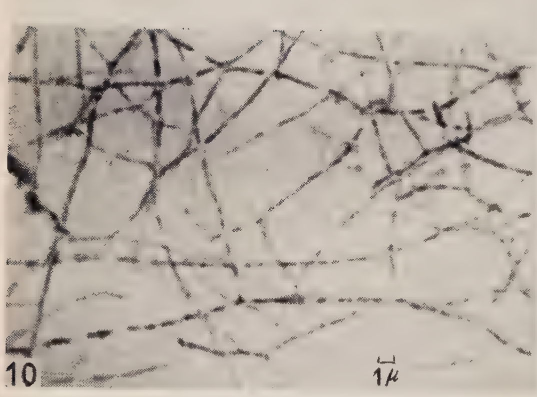
(*Facing p.* 472)

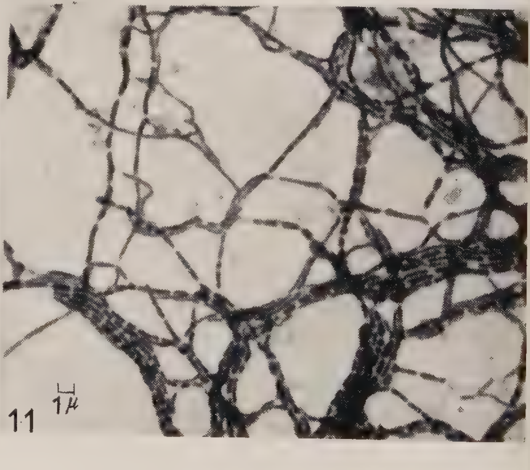












A. PROKOFIEVA-BELGOVSKAYA & L. POPOVA—CHLORTETRACYCLINE PRODUCTION BY *S. AUREOFACIENS*. PLATE 2

EYLAR, O. R. JUN. & SCHMIDT, E. L. (1959). *J. gen. Microbiol.* **20**, 473–481

A Survey of Heterotrophic Micro-Organisms from Soil for Ability to Form Nitrite and Nitrate

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SUMMARY: A total of 978 cultures of heterotrophic organisms were isolated from twelve actively nitrifying soils; each isolate was tested for ability to form nitrite or nitrate in glucose peptone broth. None of the isolates yielded substantial amounts of nitrite; concentrations found did not exceed 2 μg. nitrite-N/ml. Almost 7% of the isolates formed nitrite-N in excess of 0·2 μg./ml., while slightly over 2% yielded more than 0·5 μg./ml. Fungus isolates were the most numerous and most active nitrite producers; fifteen of the fungi formed nitrate in addition to nitrite. Concentrations of 5–45 μg. nitrate-N/ml. were recorded for the active fungi. Further isolations of fungi from ten soils of diverse properties resulted in 353 cultures but only three of these formed nitrate. One of these cultures yielded 90 μg. nitrate-N/ml. after 14 days growth in the test medium.

Most of the fungi which produced nitrate (16 of the 18 active cultures) were identified as *Aspergillus flavus*. A *Penicillium* sp., and a *Cephalosporium* sp. also formed low concentrations of nitrate. Each isolate of *A. flavus* obtained from soil proved capable of nitrate formation. Strains of *A. flavus* were encountered which did not yield nitrate, but these had been obtained from culture collections, and had been carried on artificial media for many years. Some stock cultures of *A. flavus*, several cultures of *A. flavus* and one of *A. glaucus*, freshly isolated from wheat seed, also were active nitrate producers. Ammonium sulphate and urea media supported growth of soil fungus isolates but not nitrate formation. Other organic nitrogen substrates in order of increasing effectiveness in nitrate production were: yeast extract, peptone, Protone and casein.

The possibility that autotrophic bacteria of the family Nitrobacteraceae are not the sole agents of nitrification has been considered many times. Reviews of the early literature by Barritt (1933), Stephenson (1939) and Waksman (1946) mention scattered claims for the isolation of heterotrophic micro-organisms capable of effecting nitrification reactions. Stephenson (1939) expressed what appears to be an accepted interpretation of these papers: 'Numerous instances reported in the literature of nitrification occurring in organisms growing heterotrophically have been examined, and serious objections to the technique have been made in every case.' Renewed interest in nitrification by heterotrophic micro-organisms is justified by a number of reports. Study of the transformations of pyruvic acid oxime in soil led Quastel & Scholefield (1949) to conclude that heterotrophic forms, rather than autotrophic nitrifying bacteria, oxidized the oxime to nitrite. Several bacteria, identified as belonging to two species of *Achromobacter* and one species of *Corynebacterium*, were reported later to be responsible for this transformation (Quastel, Scholefield & Stevenson, 1950, 1952). Jensen (1951) isolated three more groups of heterotrophic micro-organisms which formed nitrite from the oxime of pyruvic acid: 24 strains of *Nocardia corallina*, 1 strain of *Agrobacterium* sp., 3 strains of *Alcaligenes* sp. were listed. One *N. corallina* culture produced 40 μg. nitrite-N/ml. in 8 days with pyruvic oxime as the only nitrogen source, and 70 μg. nitrite-N/ml. in oxime + peptone medium.

A new species of actinomycete, *Streptomyces nitrificans*, was described for its ability to oxidize ethylurethane to nitrite (Schatz, Isenberg, Angrist & Schatz, 1954; Isenberg *et al.* 1954). The quantity of nitrite formed was extremely small; cultures grown for 68 days on 0·2% (w/v) ethylurethane produced only about 0·07 μg. nitrite-N/ml. Fisher, Fisher & Appleman (1956) isolated sixteen Gram-negative to Gram-variable rod-shaped soil bacteria which formed nitrite in soil extract + ammonia media. Nitrite-N was limited to a maximum of about 10 μg./ml. in 3 weeks, and the origin of the nitrite was in doubt. Several methane oxidizing bacteria were shown by Hutton & ZoBell (1953) to oxidize ammonium chloride to nitrite in a defined medium. While it is not possible to calculate with certainty from their data, it appears that about 20 μg. nitrite-N/ml. were produced during 19 days of incubation.

While a number of rather different groups of micro-organisms isolated from soil have been shown to form nitrite in pure culture, only one heterotroph has been implicated in the formation of nitrate. Schmidt (1954) reported the isolation of a soil fungus, *Aspergillus flavus*, which formed substantial concentrations of nitrate when grown in a yeast-extract + peptone + glucose broth. The nitrate-N concentration realized in some cases exceeded 35 μg./ml. in 2–3 weeks. Nitrite was also formed, but in negligible amounts.

The results of the pure culture studies cited may not be extrapolated directly to assess the contribution of heterotrophic micro-organisms to nitrification reactions in the soil. Before a realistic appraisal of their importance can be made, much more work is needed to observe the occurrence of 'heterotrophic nitrifiers' in soils, to characterize the physiological features of the organisms, to define the cultural conditions which lead to nitrite and nitrate formation, and to demonstrate the existence in soil of adequate specific substrates. The present report summarizes data obtained in a survey of the heterotrophic populations of numerous soils for the occurrence of micro-organisms which might participate in the formation of nitrite and nitrate, and something of the nature and properties of such organisms.

METHODS

Twenty-two soil samples were used in the study. The first twelve samples were field and barn-yard soils collected from sites in Minnesota shown to have high to very high nitrifying capacities (Schmidt, 1956); the remaining ten samples were selected to represent soils of widely different physical and chemical properties, and have been characterized elsewhere (Schmidt & Ruschmeyer, 1958).

Media used in the isolation and testing of soil organisms were chosen after preliminary experiments which indicated that the addition of ammonia to organic nitrogen sources did not increase the frequency with which heterotrophic nitrifying forms were obtained. Further trials suggested that direct isolation of micro-organisms from soil was as effective as isolation from enrichment cultures which had been inoculated with soil and then allowed to develop a strong nitrite test.

The procedure adopted for routine isolation of bacteria and actinomycetes involved plating of dilutions prepared from fresh soil in a medium of the following composition (%, w/v): glucose, 0·2; peptone, 0·4; yeast extract, 0·1; dibasic potassium phosphate, 0·1; magnesium sulphate, 0·05; ferrous sulphate, 0·001; manganese sulphate, 0·001; agar, 1·5. Rose bengal and streptomycin were added to this medium for plating the fungi (Martin, 1950). Every colony which appeared to be distinctive on macroscopic and microscopic examination was transferred to a slope of the isolation medium and maintained in culture until tested. Only fungi were isolated from the last ten soil samples.

Isolates were tested for ability to form nitrite and/or nitrate after growth for 14 days under static or shaken conditions in a liquid glucose peptone medium of the composition as given above, but with the yeast extract omitted. Glucose and peptone solutions were sterilized separately from the salts solution and added aseptically. Each culture was tested qualitatively for nitrite and nitrate; when either was positive, nitrite was determined quantitatively by the Griess-Ilosvay reagents and nitrate by the phenoldisulphonic method (both as outlined by Snell & Snell, 1949). Uninoculated medium was analysed in each experiment and maximum values did not exceed 0·02 μg. nitrite-N/ml. or 2·0 μg. nitrate-N/ml.

RESULTS

Studies of twelve soils known to have high nitrifying capacity resulted in the isolation and testing of 978 cultures of heterotrophic organisms. The occurrence among these isolates of forms which produced measurable amounts of nitrite or nitrate is summarized in Table 1. With 0·2 μg. nitrite-N/ml. accepted as a threshold value (10 times the maximum encountered in control media) 66 of the 978 cultures proved capable of forming nitrite. It is clear that this number of 'nitrite producers' (*c.* 7 % of the isolates) would be substantially smaller if a higher concentration of nitrite were considered; few of the cultures produced

Table 1. *Micro-organisms isolated from twelve soils of high nitrifying capacity and the incidence forms capable of producing nitrite and nitrate when grown on glucose peptone medium*

Isolates	Total number	No. which formed NO_2-N in excess of 0·2 μg./ml.	No. which formed NO_2-N in excess of 0·5 μg./ml.	No. which formed NO_3-N in excess of 5·0 μg./ml.
Actinomycetes	222	16	1	0
Bacteria	341	24	8	1
Fungi	415	26	14	15
Total	978	66	23	16

nitrite-N in excess of 0·5 μg./ml. and most of those were fungi. The fungus isolates were of further interest for, as noted in Table 1, they were almost exclusively responsible for any nitrate observed.

The production of nitrite and nitrate by the most active isolates is shown in Table 2 which gives the concentrations (as nitrogen equivalents) attained. In no instance was there substantial accumulation of nitrite; the maximum concentration realized was equivalent to 1·80 μg. nitrate-N/ml.; and this must be considered very low in comparison with nitrite formation by classical autotrophic nitrifying bacteria. Among the fungus isolates which yielded the most nitrite it is clear, from Table 2, that all save one produced nitrate in addition to nitrite. Moreover, with the exception of a single bacterial culture, the only forms which did produce nitrate were fungi.

Table 2. *Production of* NO_2-N *and/or* NO_3-N *by soil isolates grown in glucose + peptone medium, and incubated for* 14 *days in static or shaken culture*

	NO_2-N (μg./ml.)		NO_3-N (μg./ml.)	
Organism*	Static	Shaken	Static	Shaken
F-1-0	1·80	0·80	26·1	26·5
B-2-14	0·15	1·66	0·0	0·0
F-2-3	0·54	0·53	3·2	0·0
F-2-5	0·60	0·45	15·4	0·0
B-3-3	0·00	0·91	0·0	0·0
B-3-4	0·06	0·66	0·0	0·0
B-4-2	0·05	0·50	0·0	0·0
B-4-6	0·03	0·60	0·0	0·0
F-4-6	0·02	0·70	0·0	0·0
F-5-4	0·38	1·60	10·0	19·4
F-6-32	1·49	0·27	30·8	15·5
B-7-18	0·03	0·61	0·0	0·0
A-8-2	0·98	1·46	0·0	0·0
B-8-12	0·03	0·56	0·0	0·0
F-9-2	0·84	1·03	10·8	5·5
F-9-8	0·70	1·08	32·8	8·9
F-9-10	0·88	0·86	45·0	11·1
F-9-11	0·54	1·12	24·5	2·9
F-9-20	0·41	0·41	21·5	12·2
F-9-21	0·42	0·42	10·4	9·8
F-9-22	0·52	0·57	19·3	15·0
F-9-23	0·81	0·48	9·3	9·7
F-9-25	1·28	0·38	20·5	9·3
F-11-25	0·46	0·46	27·5	11·0
F-11-26	0·39	0·39	12·3	8·6
B-12-21	0·02	0·29	11·0	0·8

* A = actinomycete; B = bacteria; F = fungus. Further designations refer to soil and isolate numbers.

Nitrate production by the fungi was much more variable than nitrite production, but concentrations as high as 45 μg. nitrate-N/ml. were attained by certain of the isolates. Static culture gave nitrate concentrations usually as high and generally much higher than those obtained under shaken conditions. The one bacterium which evinced ability to produce nitrate (isolate B-12-21) was of much interest as we are not aware of any report in the literature that has associated a heterotrophic bacterium with nitrate formation. As further trials with this culture gave very erratic results, an evaluation of the isolate has been left for further experiment.

The substantial concentrations of nitrate produced by some isolates and the obvious importance of fungi in the transformation suggested that a variety of soils should be studied, with emphasis on the occurrence of fungi capable of nitrate formation. In Table 3 are presented the results of testing fungal isolates from ten soils of widely different physical and chemical features. Only three active cultures were found among the 353 isolates tested, but one of these (isolate F-16-1) yielded 90 μg. nitrate-N/ml. in static culture.

Table 3. *Active fungal isolates*

The three active fungal isolates obtained from ten soils of diverse properties, and NO_2-N and/or NO_3-N produced by each in glucose + peptone medium during 14 days incubation at 28°, in static or shaken culture. Only isolates which formed NO_2-N in excess of 0·5 μg./ml. or NO_3-N in excess of 5 μg./ml. are reported; 353 fungal isolates were studied.

	NO_2-N (μg./ml.)		NO_3-N (μg./ml.)	
Isolate	Static	Shaken	Static	Shaken
F-13-34	0·00	0·00	14·0	0·0
F-16-1	0·89	1·05	90·0	45·0
F-19-23	0·15	0·00	11·8	0·0

In all, twenty fungal isolates were found to produce nitrite and/or nitrate. A classification of these isolates is given in Table 4. Seventeen of the twenty cultures proved to be *Aspergillus flavus*. Throughout the present work it was observed that every isolate of *A. flavus* recovered from soil was capable of forming nitrate. Moreover, these data show that the very unusual property of nitrate formation was limited almost exclusively to *A. flavus*, for only two among the 751 isolates of other fungal species formed even a trace of nitrate. One isolate (F-13-34) yielded 14·0 μg. nitrate-N/ml., and isolate F-19-23 a *Penicillium*) produced 11·8 μg. nitrate-N/ml. Another *Penicillium* isolate (F-4-6) formed nitrite but not nitrate.

Included also in Table 4 are the results of experiments in which the fungal isolates were grown in media containing nitrogen sources other than peptone. The isolates grew well in all media, but neither ammonia nor urea as nitrogen sources resulted in the accumulation of nitrite or nitrate. Yeast-extract nitrogen was not as good as peptone, while results with the Protone nitrogen (predominantly proteoses) approximated those obtained in peptone media. Casein was clearly the most suitable nitrogen source for the production of nitrite and nitrate by most of the fungal isolates.

The consistent ability of soil isolates of *Aspergillus flavus* to produce nitrate, and the failure to find soil fungi other than *A. flavus* with this capacity, prompted an examination of aspergilli obtained from sources other than soil. Twenty isolates of the *A. flavus* group and a few representatives of other groups of aspergilli were gathered from a number of culture collections. Growth in static culture for 14 days in the glucose peptone medium gave the results

Table 4. *Cultures which yielded nitrite or nitrate in glucose + peptone test medium, and their nitrification products as* N *in µg./ml. in the test medium with nitrogen sources other than peptone*

Urea was added at the rate of 0·2 %, other nitrogen sources at 0·5 % and incubation was in static culture for 14 days.

		Media									
		Ammonium sulphate		Urea		Yeast extract		Protone (Difco)		Casein (Difco)	
		Amounts of nitrite or nitrate as µg. N/ml.									
Isolate	Identification	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N
F-1-0	*Aspergillus flavus*	—	—	0·01	—	0·99	13·8	0·57	15·9	1·37	46·0
F-2-3	*A. flavus*	—	—	—	—	0·24	2·9	—	—	0·11	4·0
F-2-5	*A. flavus*	—	—	0·01	—	1·04	11·6	0·77	15·5	2·28	59·0
F-4-6	*Penicillium* sp.	—	—	—	—	—	—	—	—	—	—
F-5-4	*Aspergillus flavus*	—	—	0·10	—	0·40	10·2	0·37	15·4	1·68	38·0
F-6-32	*A. flavus*	—	—	0·02	0·3	0·99	9·2	0·19	15·0	3·48	40·5
F-9-2	*A. flavus*	—	—	—	—	0·30	5·4	0·18	12·0	1·9	29·0
F-9-8	*A. flavus*	—	—	—	—	0·20	2·2	0·14	17·2	1·67	23·5
F-9-10	*A. flavus*	—	—	—	—	0·24	2·7	0·16	15·2	1·73	45·0
F-9-11	*A. flavus*	—	—	—	—	0·35	6·6	0·13	18·0	1·58	30·2
F-9-21	*A. flavus*	—	—	—	—	—	—	0·21	9·2	1·53	45·5
F-9-22	*A. flavus*	—	—	—	—	—	—	0·25	12·5	1·80	46·0
F-9-23	*A. flavus*	—	—	—	—	—	—	0·10	9·9	1·56	33·2
F-9-25	*A. flavus*	—	—	—	—	—	—	0·22	16·4	2·22	51·0
F-11-25	*A.flavus*	—	—	0·07	0·5	—	—	—	11·4	0·85	11·6
F-11-26	*A. flavus*	—	—	0·02	0·5	—	—	0·36	20·8	2·56	22·8
F-13-34	*Cephalosporium* sp.	—	—	—	—	—	2·2	—	1·5	—	—
F-16-1	*Aspergillus flavus*	—	—	—	—	—	—	—	—	—	—
F-19-23	*Penicillium* sp.	—	—	—	—	—	—	—	—	—	—
Control medium	—	0·01	0·1	0·02	0·1	0·02	2·9	0·00	3·0	0·09	3·1

listed in Table 5. Only six of the thirteen isolates of *A. flavus* which were taken from stock culture collections formed significant amounts of nitrate. It is possible that continued subcultivation on ordinary laboratory media may modify the ability of an *A. flavus* strain to form nitrate or nitrite. Culture isolates CMC 1–7 had only recently been isolated from wheat seeds and all but one of these isolates gave very high yields of nitrate-N. The results of testing a few cultures of *Aspergillus* other than *A. flavus* showed that nitrate production was not restricted to the *flavus-oryzae* group. The fresh isolate of *A. glaucus* formed nitrate in quantity comparable to that produced by the fresh isolates of *A. flavus*. It does not seem likely, however, that nitrate production is a common feature of aspergilli outside of the *flavus-oryzae* group, for it was observed that the 751 fungi isolated from soil in this study included a number of *Aspergillus* species and only the *A. flavus* isolates proved to be active.

Table 5. *Formation of nitrite and nitrate by Aspergillus cultures not obtained directly from soil*

Cultures incubated statically in glucose + peptone medium for 14 days.

Isolate	Source	Yield as μg. N/ml.	
		NO_2^--N	NO_3^--N
A. flavus 9807	Upjohn Co. laboratories	0·30	4·9
A. flavus 10124		0·15	4·6
A. flavus 9296		0·05	3·1
A. flavus 9170		0·09	3·5
A. flavus 871	Quartermaster collection	0·68	37·1
A. flavus 4m		0·33	14·0
A. flavus 63c		0·31	27·0
A. flavus 380		0·65	18·4
A. flavus 301	University of Minnesota	0·28	16·7
A. flavus 453	U.S.D.A. Peoria, Ill.	0·29	14·6
A. flavus 489		0·09	1·0
A. flavus 625		0·04	1·0
A. flavus 2222		0·35	11·7
A. flavus CMC1	Wheat seed, University of Minnesota	0·60	13·0
A. flavus 2		0·53	81·0
A. flavus 3		0·58	93·0
A. flavus 4		0·41	41·4
A. flavus 5		0·41	80·0
A. flavus 6		0·46	81·0
A. flavus 7		0·51	78·7
A. glaucus CMC8		0·31	52·0
A. oryzae 692	U.S.D.A. Peoria, Ill.	0·05	1·5
A. terreus 1960		0·04	1·5
A. fumigatus 1986		0·04	1·5
A. niger 328		0·40	1·5
Control medium		0·04	1·5

DISCUSSION

None of the 1331 soil isolates examined in this survey formed nitrite in significant concentrations; certainly none approached the standards cited for *Nitrosomonas* (Meiklejohn, 1953) or the concentrations achieved by the more active *Nocardia* of Jensen (1951). Less than 2 % of all isolates formed nitrite-N in excess of 0·5 μg./ml., while approximately 5% formed nitrite-N in excess of 0·2 μg./ml. In the first twelve soils examined, where all groups were studied, 27% of the actinomycetes, 26% of the bacteria, and 17% of the fungi formed at least a measurable amount of nitrite. The results of these experiments suggest that very limited nitrite formation may be a potentiality of a sizeable and diverse segment of the soil microflora, but that heterotrophic forms capable of substantial degrees of nitrite production either occur rarely in soil or were not disclosed by the procedures used.

Nitrate formation by heterotrophic organisms, in contrast to nitrite formation, was observed at substantial concentrations and was limited to a specific segment of the soil microflora. *Aspergillus flavus* merits special attention in any consideration of nitrate production. This fungal species is widely distributed in soils (Thom & Raper, 1945), was the first heterotrophic organism reported to produce nitrate (Schmidt, 1954), and accounted for nearly all instances of nitrate formation observed in this survey. One or more isolates of *A. flavus* were obtained from eight of the twenty-two sites studied, and isolation was made more commonly from the twelve soils of high nitrifying capacity than from ten other sites selected for other properties. Each *A. flavus* isolate obtained from soil proved capable of some nitrate formation in the test medium. Cultures of *A. flavus* demonstrated considerable variability in nitrate production whether obtained from soil (Table 4) or from other sources (Table 5). The nature of the nitrogen source, the aeration of the medium, and the history of subculture, all apparently affected the amount of nitrate produced.

The simple forms of nitrogen such as ammonia and urea allowed for good growth but no nitrate formation, while more complex nitrogenous materials were clearly superior as substrates for nitrate production (Table 4). It is possible that nitrate formed by *A. flavus* actually is derived from ammonia as in autotrophic nitrification, but that synthesis of the appropriate enzymes requires a complex medium; Schmidt (1954) showed that ammonia is formed along with nitrate in peptone media. Information as to the nature of specific components of the complex nitrogen sources that are a prerequisite to nitrate formation will help assess the importance of *A. flavus* as a nitrate producer in mixed culture in soil. Attempts to identify such active components are in progress.

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REFERENCES

Barritt, N. W. (1933). The nitrification process in soils and biological filters. *Ann. appl. Biol.* **20**, 165.

Fisher, T., Fisher, E. & Appleman, M. D. (1956). Nitrite production by heterotrophic bacteria. *J. gen. Microbiol.* **14**, 238.

Hutton, W. E. & ZoBell, C. E. (1953). Production of nitrite from ammonia by methane oxidizing bacteria. *J. Bact.* **65**, 216.

Isenberg, H. D., Schatz, A., Angrist, A. A., Schatz, V. & Trelawny, G. S. (1954). Microbial metabolism of carbamates. II. Nitrification of urethan by *Streptomyces nitrificans*. *J. Bact.* **68**, 5.

Jensen, H. L. (1951). Nitrification of oxime compounds by heterotrophic bacteria. *J. gen. Microbiol.* **5**, 360.

Martin, J. P. (1950). Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. *Soil Sci.* **69**, 215.

Meiklejohn, J. (1953). The nitrifying bacteria: A review. *J. Soil Sci.* **4**, 59.

Quastel, J. H. & Scholefield, P. G. (1949). Influence of organic nitrogen compounds on nitrification in soil. *Nature, Lond.* **164**, 1068.

Quastel, J. H., Scholefield, P. G. & Stevenson, J. W. (1950). Oxidation of pyruvic oxime by soil organisms. *Nature, Lond.* **166**, 940.

Quastel, J. H., Scholefield, P. G. & Stevenson, J. W. (1952). Oxidation of pyruvic oxime by soil organisms. *Biochem. J.* **51**, 278.

Schatz, A., Isenberg, H. D., Angrist, A. A. & Schatz, V. (1954). Microbial metabolism of carbamates. I. Isolation of *Streptomyces nitrificans*, n.sp., and other organisms which grow on urethan. *J. Bact.* **68**, 1.

Schmidt, E. L. (1954). Nitrate formation by a soil fungus. *Science*, **119**, 187.

Schmidt, E. L. (1956). Soil nitrification and nitrates in waters. *Publ. Hlth Rep.* **71**, 497.

Schmidt, E. L. & Ruschmeyer, O. R. (1958). Cellulose decomposition in soil burial beds. I. Soil properties in relation to cellulose degradation. *Appl. Microbiol.* **6**, 108.

Snell, F. D. & Snell, C. T. (1949). *Colorimetric Methods of Analysis*, vol. II, third edition. New York: D. van Nostrand.

Stephenson, M. (1939). *Bacterial Metabolism*, second edition. London: Longmans, Green and Co.

Thom, C. & Raper, K. B. (1945). *A Manual of the Aspergilli*. Baltimore, Md., U.S.A.: Williams and Wilkins.

Waksman, S. A. (1946). Sergi Nikolaevitch Winogradsky. *Soil Sci.* **62**, 197.

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Purification and Properties of a Polymetaphosphatase from *Corynebacterium xerosis*

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SUMMARY: An enzyme which splits high molecular weight polymetaphosphate (PMP) to orthophosphate has been extracted from *Corynebacterium xerosis*, partially purified and some of its properties studied. A method for the preparation of bacterial PMP labelled with ^{32}P is described. The enzyme hydrolyses bacterial or synthetic PMP at the same rate. No formation of short-chain phosphate polymers was detected during the reaction and the enzyme did not attack metaphosphates smaller than hexametaphosphate. It does not transfer phosphate from PMP to ADP to form ATP, nor does it synthesize PMP from orthophosphate (Pi) in the presence of ATP or other nucleotides. All the metal ions tested, including Mg^{++}, inhibited the enzyme. Sodium ethylenediaminetetraacetate (EDTA; 0·2 mM) stimulated the rate of reaction; higher concentrations inhibited. The significance of polymetaphosphate and enzymes which split it in micro-organisms is briefly discussed.

Enzymes which split polymetaphosphate (PMP) to orthophosphate (Pi) occur in extracts of a variety of micro-organisms including bacteria, yeasts and moulds (Mann, 1944; Kitasato, 1928). Most of the earlier observations were, however, made with polymetaphosphatases which split metaphosphates of low molecular weight, e.g. trimetaphosphate, tetrametaphosphate and hexametaphosphate. More recently Malmgren (1952) studied the breakdown of very high molecular weight PMP preparations by extracts from *Aspergillus niger*, *Penicillium expansum* and some other organisms. By measuring the decrease in viscosity and the amount of orthophosphate formation for 2 days at pH 5·3 he concluded that low molecular weight compounds such as tetrametaphosphate and pentametaphosphate were the major end-products. More recently, Kornberg, Kornberg & Simms (1956) prepared an enzyme from *Escherichia coli* which could synthesize PMP from adenosine triphosphate (ATP); an unidentified primer was also necessary to initiate the reaction. Kornberg (1957) showed that this enzyme also catalysed the reverse reaction, i.e. synthesis of ATP from PMP and adenosine diphosphate (ADP). A similar enzyme has also been slightly purified from *Corynebacterium diphtheriae* (Kornberg, 1957). The aim of the present investigation was to purify the polymetaphosphatase (PMP-ase) from *C. xerosis* and to study some of its properties; a relatively simple assay system has been devised. We were also interested in finding out whether this enzyme catalyses the transfer of orthophosphate (Pi) from PMP to a suitable substrate such as ADP, besides breaking PMP to Pi, and whether PMP could directly phosphorylate other compounds, such as glucose, without the intermediation of the ADP–ATP system.

METHODS

Organism. The organism used, *Corynebacterium xerosis*, was originally National Collection of Type Cultures strain no. 7243. The organism was maintained on agar slopes of the medium used for growth (see below).

Growth of organism. Corynebacterium xerosis was grown in rectangular enamelled metal trays (15 in. × 10 in. × 1½ in.) with tight-fitting lids. The plates were sterilized in an oven at 110–150° for 3 hr. The medium added to each plate had the following composition: Tryptone (Oxo Ltd.) 10 g.; glucose 10 g.; $Na_2HPO_4.2H_2O$ (British Drug Houses Ltd.) 8 g.; KH_2PO_4 (B.D.H.) 4 g.; NaCl (A.R.) 4 g.; yeast extract (Oxo Ltd.) 6 g.; water to 400 ml. The pH of the medium was 6·8–7·0. Eight g. of agar-agar (Bacto Difco Co.) were added, the medium sterilized in an Erlenmeyer flask by heating at 10 lb./sq.in. for 10 min. and then poured into the sterilized plate. Usually five plates were prepared in this way. The plates were allowed to cool until the agar solidified and the inoculum from agar slopes was spread on the medium with a sterilized stainless-steel spreader. The plates were incubated at 37° for 18 hr. After this time, the cells had fully developed metachromatic granules and were harvested from the plates with ice cold 0·15 M-NaCl; glass beads were used for loosening the organism from the agar. The suspension was passed through glass wool in order to remove lumps of agar, centrifuged in the International refrigerated centrifuge at 1000 *g*. for 20 min. and the organisms washed twice with cold 0·15 M-NaCl. The yield of organism prepared in this way was usually about 10 g wet wt./plate. The organisms were immediately processed for the extraction of the enzyme or stored in the deep freeze at −15°.

Preparation of extracts containing enzymes. Organisms (50–60 g.) were made into a thick paste by adding 15 ml. cold 0·15 M-NaCl and crushed in a Hughes (1951) bacterial press with a cylinder of 1½ in. bore. The crushed material was homogenized with 60 ml. cold 0·15 M-NaCl in a Potter type glass homogenizer (Potter & Elvehjem, 1936). The final suspension, when stained with Albert stain (Mackie & McCartney, 1945) and examined under the microscope, showed very few intact cells.

Preparation of ^{32}P *labelled PMP* (PM^{32}P). *Bacterial polymetaphosphate.* PM^{32}P was extracted from *Corynebacterium xerosis* grown on the medium described above, to which 1 mc. of carrier-free ^{32}P orthophosphate (Radiochemical Laboratory, Harwell) was added. The washed organisms were extracted with 5 vol. 10 % (w/v) trichloroacetic acid (TCA) at room temperature and constantly shaken for 5 hr. The extraction of PM^{32}P was complete in that time (see Table 1). The suspension was centrifuged, the supernatant fluid neutralized with NaOH and brought to pH 3·5 by adding 5 M-acetate buffer (pH 3·5). PM^{32}P was precipitated by the addition of 1 ml. saturated $BaCl_2$ solution. The flask was thoroughly shaken and left at 0–2° overnight. The precipitate was collected by centrifugation and washed twice with acetate buffer (pH 3·5) and once with absolute ethanol. It was then treated with Amberlite-IR 120 resin (H^+ form) in order to remove Ba^{++}, neutralized and dialysed against distilled water at 0–2°. The final solution was made up to

20 ml. giving 25,000 counts/min. (CPM) and containing 3·5 μmole P/ml. Approximately 0·25 % of the ^{32}P added to the growth medium was recovered in $PM^{32}P$. The resulting product was very viscous. Mixed with solutions of toluidine blue, it showed metachromasy. The absorption maximum shifted from 625 to 530 mμ. It was precipitated by albumin in 3·5 % (w/v) perchloric acid, and did not move on paper chromatography, with various solvents, and could not easily be removed from the anion exchange resins except by hydrolysis. The Ba^{++} precipitate conformed to the formula $(Ba(PO_3)_2)_n$ when analysed for P and Ba^{++}. All the phosphorus in the preparation was hydrolysed when treated with N-HCl at 100° for 7 min. (We have used the term polymetaphosphate in the usual sense to describe the high molecular weight phosphate polymers of this type, although no details are known about the precise structure.)

Table 1. *The extraction of PMP from* Corynebacterium xerosis *by 10 % (w/v) trichloroacetic acid solution*

Organism (20 g. wet wt.) was washed, suspended in 5 vol. of 10 % (w/v) TCA and shaken at room temperature for the times stated. The precipitate was removed by centrifugation and metaphosphate determined in the supernatant fluids as described in the text.

Time of extraction (hr.)	Metaphosphate phosphorus in extract (mg.)
0·5	1·8
1·0	3·0
1·5	4·9
2·5	7·7
4·5	13·3
9·0	14·1

In contrast to the findings with yeast (Wiame, 1949; Katchman & Van Wazer, 1954; Juni, Kamen, Reiner & Spiegelman, 1948), moulds (Belozersky & Kulaev, 1957; Krishnan, Damle & Bajaj, 1957) and certain mycobacteria (Mudd, Yoshida & Koike, 1958; Winder & Denneny, 1957) no 'acid-soluble' PMP was detected in *Corynebacterium xerosis*. When organisms were extracted with 5 % TCA at 0–2° for 1 hr. and the extract was treated with Ba^{++} at pH 3·5, no precipitate was formed.

Chemical synthesis of $PM^{32}P$. The method of Pfanstiel & Iler (1952) as modified by Kornberg (1957) for small-scale preparation was used for preparing synthetic $PM^{32}P$. To 2 ml. of M-KH_2PO_4 in a platinum crucible 200 μc. of carrier-free ^{32}Pi was added and the pH was adjusted to 4·5 by adding N-KOH. The solution was evaporated gently on a sand bath, 2 ml. distilled water added from the sides of the crucible and the solution again evaporated. The crucible was then placed in a muffle furnace, the temperature of the furnace was raised to 770° over a period of 2 hr. and maintained at 770–780° for 1 hr. The crucible was then removed from the furnace and allowed to cool. The residue was washed twice with ice-cold distilled water and then treated with Amberlite-IR 120 (Na^+ form). The mixture was stirred vigorously for 2 hr. at 0–2°. After this, the resin was removed by centrifugation and the resulting viscous solution was dialysed against demineralized water at 0–2° for 24 hr. The yield of PM^{32}P on the basis of P content and radioactivity measurements was 50–60 %. The product had properties similar to those of bacterial PM^{32}P.

Chemicals. Adenosine triphosphate (sodium salt) and adenosine diphosphate (ADP) were products of the Schwarz Laboratories, Inc., U.S.A. ADP (Ba salt) was converted into the Na$^+$ form by shaking with Amberlite IR-120 (H$^+$ form) and neutralized with NaOH. Adenosine-5′-monophosphate (AMP; acid) was a Waldhof Type I product of Zellstoffabrik, Wiesbaden, Germany, and was neutralized before use. Potassium polymetaphosphate, sodium hexametaphosphate, sodium tetrametaphosphate and sodium trimetaphosphate were kindly given by Messrs Albright and Wilson Ltd. Pancreatic ribonuclease, deoxyribonuclease and protamine sulphate were obtained from Messrs Light and Co. Ltd.

As the mol. wt. of PMP was not known, the molarity of PMP solutions is given in terms of orthophosphate (Pi) liberated after hydrolysis with N-HCl at 100° for 7 min. The concentration of hexametaphosphate is expressed similarly for the same reason.

Chemical estimations. Phosphorus was estimated by the method of Berenblum & Chain (1938). The sample was extracted as usual in isobutanol and the volume made up to 10 ml. with ethanol. Before developing the colour with $SnCl_2$ the sample was counted in a GM liquid counter tube (type M6) to estimate ^{32}P. A suitable number of counts was taken to ensure a standard error of $\pm 2\,\%$.

Barium was estimated gravimetrically as $BaSO_4$, protein by the biuret method (Gornall, Bardawill & David, 1949) and also by the spectrophotometric method of Kalckar (1947) when the nucleic acid content of the solution was low. Total N was estimated by the micro-Kjeldahl method.

Measurement of enzyme activity. The assay measured the amount of ^{32}Pi liberated from PM^{32}P under standard conditions. In the initial work the assay system contained 0·1 ml. $MgCl_2$ (2·0 mM), 0·1 ml. 2-amino-2-hydroxymethyl propane-1:3 diol (tris) + maleate buffer (0·1 M, pH 7·0), 0·1 ml. PM^{32}P (0·5 μ-mole, 10,000–20,000 CPM/μmole), enzyme, and water to final volume 0·5 ml. This system was later altered by omitting $MgCl_2$ and substituting 0·1 ml. of sodium ethylenediaminetetra-acetate (EDTA; 1 mM). The mixture was incubated at 37° for 20 min. and then 0·5 ml. of cold 7 % (w/v) perchloric acid and 1·0 ml. 0·1 % (w/v) bovine serum albumin (Armour) was added. The mixture was stirred and placed on ice for 5 min., centrifuged at 0–2° at 10,000 *g* for 5 min., and a suitable sample of the supernatant fluid taken for extraction of ^{32}Pi by the method of Berenblum & Chain (1938) and counted. One unit of the enzyme is defined as that amount which catalyses the breakdown of 0·001 μmole of non-dialysable PMP to orthophosphate in 20 min. at 37°.

Purification of the enzyme. Step I. Nuclease treatment. The crushed organisms gave a very viscous suspension after homogenizing. Before attempting any purification, the suspension was treated with nucleases, which resulted in a decrease in viscosity. To 100 ml. of the thick suspension was added approximately

1 mg. each of ribonuclease and deoxyribonuclease (Light and Co. Ltd.), 20 μmole-$MgCl_2$ and tris buffer (pH 7·0). The suspension was incubated at 37° for 20 min. with occasional shaking, after which it was immediately cooled in ice and centrifuged in the Spinco Model 'L' ultracentrifuge at 50,000 *g* for 20 min.; 60–70 % of the activity of original extract was then present in the supernatant fluid. The residue was discarded.

Step II. Ammonium sulphate treatment I. Saturated $(NH_4)_2SO_4$ solution (neutralized with NaOH) was added to the supernatant fluid from step I at 0–2°. The $(NH_4)_2SO_4$ addition was done with constant mechanical stirring until a final concentration of 60 % saturation was reached. The mixture was kept in ice for 10 min. and centrifuged at 0–5° in the International refrigerated centrifuge. The supernatant fluid, which was inactive, was discarded and the bulk of the activity was recovered in the residue which was dissolved in tris + maleate buffer (pH 7·0) to give a final volume of 23 ml.

Table 2. *Summary of the enzyme-purification procedure*

Step no.	Volume (ml.)	Enzyme activity (units/ml.)	Protein concn. (mg./ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. protein)
1. Nuclease-treated crushed organisms	90	1260	42·20	113,400	3798	29
50,000 *g* supernatant fluid	75	1080	20·10	81,000	1507	54
2. $(NH_4)_2SO_4$ I	23	3360	40·90	77,280	941	82
3. Protamine sulphate $(NH_4)_2SO_4$ II	28	2560	4·90	71,680	137	522
4. $(NH_4)_2SO_4$ III	25	2344	2·02	58,600	51	1132
5. Ethanol	20	1073	0·38	21,460	7·6	2823

Step III. Protamine sulphate treatment. Protamine sulphate solution (20 mg./ml.; pH 5·0) was slowly added to the solution from step II in the cold and with constant stirring. The precipitate was centrifuged off and the supernatant fluid examined for absorption at 260 and 280 mμ. The addition of protamine sulphate was continued until the E 280/E 260 ratio was more than 0·75, the reason being that at ratios higher than this the amount of nucleic acid remaining in the supernatant fluid was very small and did not interfere with further fractionation. A total of 15 ml. of the solution of protamine sulphate (equiv. 300 mg.) was added and the precipitate was removed by centrifugation. The supernatant fluid was diluted with cold distilled water to 400 ml. with constant stirring, and the bulky precipitate formed was centrifuged off and discarded. To the supernatant fluid was added 152 g. solid $(NH_4)_2SO_4$ and the precipitate was collected and dissolved in 20 ml. water ($(NH_4)_2SO_4$(II)). About 90–95 % of the activity from step II was present in the final solution.

Step IV. $(NH_4)_2SO_4$ *fractionation III.* The above solution was subjected to $(NH_4)_2SO_4$ fractionation. Saturated $(NH_4)_2SO_4$ solution was added to give a saturation of 28 % and the precipitate was centrifuged off and discarded. Saturated $(NH_4)_2SO_4$ was added to the supernatant fluid to bring the concentration to 54 % and the precipitate which formed was collected and redissolved in 25 ml. water ($(NH_4)_2SO_4$(III)).

Step V. Ethanol fractionation. The $(NH_4)_2SO_4$ (III) fraction was further purified by reprecipitation with absolute ethanol at −15°. The fractionation was carried out in a salt ice bath maintained at $-15 \pm 1°$, and the fractions were collected in the refrigerated centrifuge at −15°. The fraction appearing between 60 and 75 % (v/v) ethanol in water contained most of the activity and was collected. Active material was extracted with tris buffer (pH 7·0). In some experiments, where concentrated enzyme preparation was required, the final solution was brought to a 50 % saturation with $(NH_4)_2SO_4$ and the residue dissolved in a small amount of water without loss of activity. The enzyme preparation thus obtained was dialysed against 0·1 M-$(NH_4)_2SO_4$ at pH 7·0 and 0–2° for 24 hr. The overall purification, on a protein basis, was approximately 100 times.

RESULTS

Specificity. The purified enzyme did not hydrolyse trimetaphosphate, tetrametaphosphate, pyrophosphate, ATP or glucose-6-phosphate. Hexametaphosphate (Graham's salt) was broken down almost as rapidly as PMP. Bacterial PMP was hydrolysed as rapidly as the synthetic PMP (Table 3).

Table 3. *Action of PMP-ase on various substrates*

The reaction mixture consisted of $MgCl_2$ solution 0·1 ml. (0·2 μmole); tris + maleate buffer (pH 7·0) 0·1 ml.; enzyme solution 0·1 ml. (780 units); substrate solution, 0·1 ml.; water, 0·1 ml. The mixture was incubated at 37° for 20 min. and the reaction stopped by adding 0·5 ml. 7 % (w/v) perchloric acid. The mixture was centrifuged and orthophosphate (Pi) estimated in the supernatant fluid by the method of Berenblum & Chain (1938). A blank was run for the enzyme and for each substrate.

	Amount added		Pi formed (μmole)		
Substrate	Substrate (μmole)	Total Pi in substrate (μmole P)	Observed	Blank	Net
Trimetaphosphate	0·5	1·5	0·14	0·14	0
Tetrametaphosphate	0·5	2·0	0·14	0·14	0
Pyrophosphate	1·00	2·0	0·22	0·23	0
ATP	1·00	3·0	0·25	0·26	0
Glucose-6-PO_4	0·50	0·5	0·08	0·08	0
Hexametaphosphate	—	1·58	1·29	0·46	0·83
Polymetaphosphate (synthetic)	—	1·01	0·86	0·08	0·78
Polymetaphosphate (bacterial)	—	0·05	0·46	0·07	0·39

Addition of ATP, ADP or AMP to the complete reaction mixture did not significantly affect the breakdown of PMP to orthophosphate (Table 4). When ADP, glucose and hexokinase were added, there was no decrease in the counts appearing in the orthophosphate fraction and no incorporation into the acid stable P fraction. This clearly indicates that the enzyme is not capable of transferring phosphate from PMP to ADP or AMP (cf. Kornberg, 1957).

Table 4. *Effect of adenine nucleotides on PMP-ase activity*

The reaction mixture consisted as usual of tris buffer, Mg^{++}, $PM^{32}P$ and enzyme solution. Following additions were made and enzyme activity measured as usual.

Addition	Final concn. (mM)	Counts/min. in ^{32}Pi
None	—	3491
ATP	2·0	3532
ADP	4·0	3505
AMP	2·0	3545
Glucose	10·0	3502
ADP	2·0	
Hexokinase	0·05 mg. protein	

Kinetics of the enzyme

pH *effect and stability.* The pH dependence of the enzyme was studied in 0·1 M-tris + maleate buffer at pH 5–8. The optimum pH value was at pH 7·0 and there was very little activity at pH 6·0 (Fig. 1). The enzyme was completely inactivated by incubation at 50° for 20 min. Incubation at 37° for 20 min. resulted in a 10 % loss in activity as compared with the activity at 0° (Fig. 2). There was no loss in activity when the purified enzyme was stored at −15° from 5 to 6 weeks.

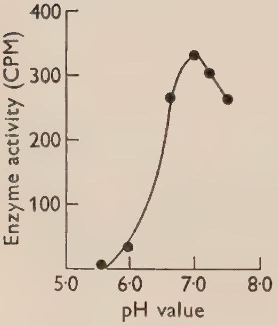

Fig. 1

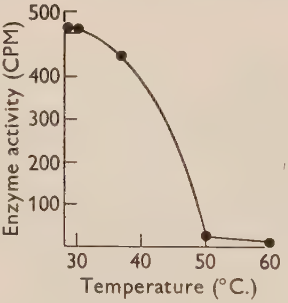

Fig. 2

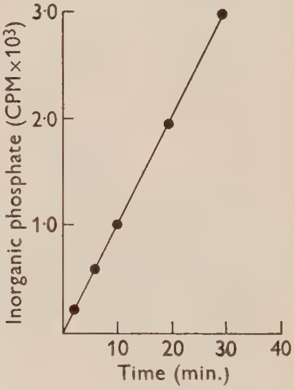

Fig. 3

Fig. 1. Effect of pH value on enzyme activity. The reaction mixture contained purified dilute enzyme 50 units; $PM^{32}P$, 0·5 μmole-P; $MgCl_2$, 0·2 μmole; tris + maleate buffer (10 μmole); water to 0·5 ml. Enzyme activity was measured as usual after 20 min. incubation at 37°.

Fig. 2. Effect of temperature on enzyme activity. Assay mixture contained: purified enzyme 200 units; $PM^{32}P$, 0·5 μmole; tris + maleate buffer (pH 7·0), 10 μmole; $MgCl_2$, 0·2 μmole; water to 0·5 ml. Before adding to the assay system the enzyme preparation was first incubated for 20 min. at the different temperatures and then assayed for activity as usual.

Fig. 3. Rate of enzyme reaction with time. The reaction mixture contained: purified enzyme 320 units; $PM^{32}P$, 0·5 μmole; tris + maleate buffer (pH 7·0), 10 μmole; $MgCl_2$, 0·2 μmole; water to 0·5 ml. Reaction was stopped after different intervals of time and activity measured as usual.

Effect of substrate concentration and rate of reaction. The progress of the reaction was proportional with time during a 30 min. period (Fig. 3). The rate of reaction increased approximately linearly with low substrate concentrations up to a final concentration of 0·30 mM (Fig. 4*a*). Further increase in substrate concentration did not increase the rate of orthophosphate formation linearly. The *Km* value for the PMP-ase as determined from the Burk-Lineweaver plot is $7{\cdot}7 \times 10^{-4}$ M (Fig. 4*a, b*), the molarity being expressed as inorganic orthophosphate added as PMP.

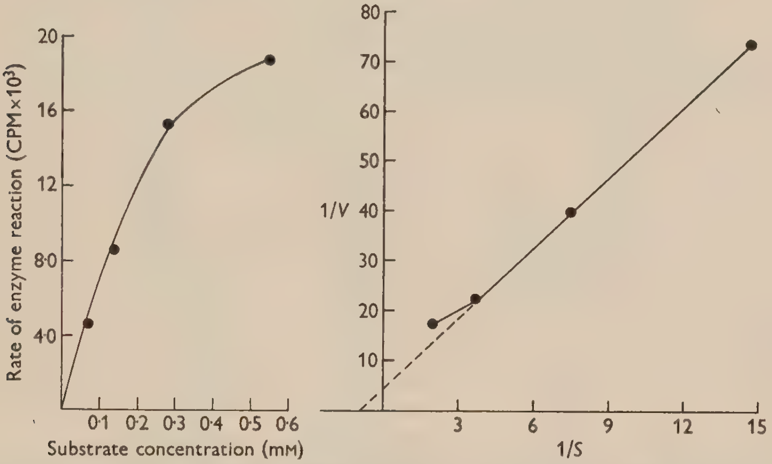

Fig. 4*a, b*. Initial rate of reaction at different substrate concentrations. The reaction mixture contained: purified enzyme previously dialysed against EDTA, 80 units; tris + maleate buffer (pH 7·0), 10 μmole; EDTA, 0·2 μmole; different amounts of PM^{32}P; water to 0·5 ml. Enzyme activity was measured as usual after 20 min. incubation at 37°. The data is plotted according to Lineweaver & Burk (1934) in *b*.

When ^{32}Pi was incubated in the presence of ATP instead of PM^{32}P, all the radioactivity was recovered in the acid soluble inorganic phosphate fraction. As such, no PM^{32}P was synthesized and the reaction was not reversible under these conditions.

Effect of metal ions and EDTA on PMP-ase activity. The effect of metal ions, EDTA and some inhibitors on the purified enzyme is shown in Table 5. EDTA stimulated the enzyme appreciably at a final concentration of 0·5 mM. Ammonium molybdate and ammonium vanadate had a slightly stimulatory effect. All the other metal ions tried, namely Mg^{++}, Ca^{++}, Fe^{++}, Mn^{++}, Co^{++}, Zn^{++} and Cu^{++} inhibited the enzyme to different extents. Uranyl acetate (0·1 mM) almost completely inhibited the polymetaphosphatase activity.

Dialysis against water, tris buffer (0·1 M), pyrophosphate buffer (0·1 M) or EDTA (0·02–0·05 M) at pH 7·0 and 0–2° completely inactivated the enzyme. Dialysis against 0·1 M-$(NH_4)_2SO_4$ under the above conditions did not affect the enzyme activity. In an attempt to remove metal ions the enzyme was dialysed, with continuous stirring, against 0·1 M-$(NH_4)_2SO_4$ + 50 mM-EDTA (pH 7·0) and 0–2° for 24 hr. EDTA was removed by dialysis against 0·1 M-$(NH_4)_2SO_4$ for 40 hr. The effect of different concentrations of Mg^{++} and EDTA was tried on

Table 5. *Effect of metal ions and inhibitors on the PMP-ase activity*

The reaction mixture contained: tris + maleate buffer (pH 7·0) 0·1 ml.; purified enzyme solution 0·1 ml.; $PM^{32}P$, 0·1 ml.; other additions as shown below.

Addition		
Substance	Final concentration (mM)	Counts/min. in Pi fraction
No addition	—	3102
EDTA	0·4	3807
Ammonium molybdate	0·4	3422
Ammonium vanadate	0·1	3218
$MnCl_2$	0·58	2737
$FeSO_4$	0·55	2722
$CuCl_2$	0·58	2642
$MgCl_2$	0·4	2516
$CaCl_2$	0·4	2232
$ZnCl_2$	0·4	1123
$Co(NO_3)_2$	0·4	609
Uranyl acetate	0·1	151
Sodium arsenate	1·00	3152

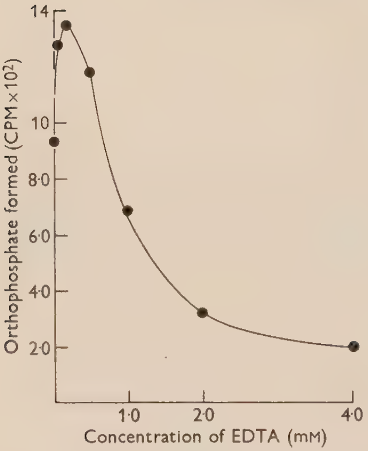

Fig 5

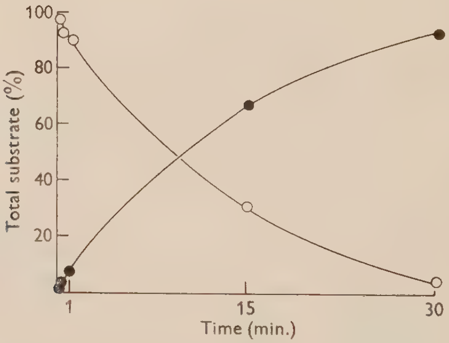

Fig. 6

Fig. 5. The effect of EDTA on the activity of purified enzyme. The reaction mixture contained: purified enzyme previously dialysed against EDTA 105 units; $PM^{32}P$, 0·5 μmole; tris + maleate buffer (pH 7·0), 10 μmole; different concentrations of EDTA; water to 0·5 ml. Enzyme activity was measured as described in the text.

Fig. 6. The release of orthophosphate by PMP-ase action on $PM^{32}P$ compared to non-dialysable $PM^{32}P$ remaining. Composition of the reaction mixture was as given in the text (p. 491). ●—●, ^{32}Pi formed; ○—○, non-dialysable ^{32}Pi left.

the enzyme preparation dialysed in this way. It was clear (Table 6) that EDTA at low concentration stimulated the enzyme, whereas when the concentration was increased, EDTA had an inhibitory effect (Fig. 5). Mg^{++} inhibited the purified enzyme even at low concentrations. When EDTA was added in concentrations sufficient to complex added metals it annulled their inhibitory effects.

Mechanism of PMP-ase action

Estimation of orthophosphate and non-dialysable radioactivity in the reaction mixture. Seven tubes were set up as follows: $MgCl_2$ 0·1 ml. (0·2 μmole); tris-maleate buffer 0·1 ml. (0·1 M, pH 7·0); $PM^{32}P$, 0·1 ml. (3062 CPM); purified enzyme 0·1 ml. (600 units); water, 0·1 ml. The reaction was stopped after suitable intervals by adding 0·5 ml. cold 7 % (w/v) perchloric acid, the mixture centrifuged and the clear supernatant fluid taken in another tube kept in ice. A sample (0·4 ml.) of the supernatant fluid was taken for ^{32}Pi estimation and another sample (0·25 ml.) for dialysis. The samples were dialysed overnight in 15 l. of demineralized water at 0–2° and the radioactivity remaining in the non-dialysable solution was counted. The results are given in Table 7 and Fig. 6.

Table 6. *Effect of different concentrations of EDTA and* Mg^{++} *on PMP-ase activity*

The reaction mixture contained: tris + maleate buffer (pH 7·0) 0·1 ml.; purified enzyme solution, 0·1 ml.; $PM^{32}P$, 0·1 ml.; and varying amounts of EDTA or Mg^{++} as shown below.

Addition		
Substance	Final concentration (mM)	Counts/min. in Pi fraction
None	—	940
EDTA	0·05	1287
EDTA	0·20	1350
EDTA	0·50	1175
EDTA	1·00	684
EDTA	2·00	326
EDTA	4·00	199
Mg^{++}	0·05	802
Mg^{++}	0·20	613
Mg^{++}	0·50	425
Mg^{++}	2·00	307

Table 7. *The amounts of* ^{32}Pi *formed and non-dialysable radioactive material (as counts) left after PMP-ase action for different times*

Composition of the reaction mixture as given in the text.

Period of incubation (min.)	*a*. Counts in Pi fraction (calculated in the total reaction mixture) (CPM)		*b*. Counts after dialyses (CPM)		Recovery	
	Observed	% of total in $PM^{32}P$	Observed	% of total in $PM^{32}P$	Total CPM $(a+b)$	% of total counts in $PM^{32}P$ taken
0	60	1·9	2939	95·9	2999	97·9
0·25	115	3·7	2830	92·4	2945	96·1
1	240	7·8	2737	89·4	2977	97·2
15	2032	66·8	942	30·9	2972	97·2
30	2832	92·1	128	4·1	2951	96·2
60	3053	99·7	16	0·5	3069	100·2
300	3070	100·2	0	0	3070	100·2

It is clear from the results of the above experiment that when PMP was broken down no dialysable intermediates other than Pi were formed. Very little PMP was left after incubation for 1 hr. and almost all of it was recovered as Pi.

Chromatography of the reaction mixture. To confirm the conclusion of the above experiment, the reaction mixtures after different intervals of time were chromatographed. Five tubes were assembled as shown in Table 8. The reaction was stopped by adding 0·1 ml. cold 21 % (w/v) perchloric acid and the mixture centrifuged. A sample of the supernatant fluid (0·1 ml.) was spotted on Whatman no. 4 filter-paper for ascending chromatography as described by Berg (1958). Trimetaphosphate, tetrametaphosphate, PMP and Pi were run as markers. The composition of the solvent was methanol 50 ml., *n*-butanol 10 ml., water 22 ml., aqueous concentrated ammonia (sp.gr. 0·880) 18 ml., titrated with 25 % (w/v) formic acid to pH 11·4. The solvent was run for 16 hr. at room temperature, dried and sprayed with Hanes & Isherwood phosphate spray reagent (Hanes & Isherwood, 1949). The spot with the same R_f value as tetrametaphosphate was present in the enzyme blank and samples 3–5, and did not have any radioactivity. This spot was light blue as compared to the phosphate spots (including tetrametaphosphate) which were dark blue. The spot did not contain any phosphorus, as determined by the method of Berenblum & Chain (1938) after ashing, and has not yet been identified.

Table 8. *Chromatography of the reaction mixtures*

The tubes had $MgCl_2$ solution, 0·1 ml. (0·2 μmole); tris + maleate buffer (pH 7·0), 0·1 ml., $PM^{32}P$, 0·1 ml. (2980 CPM, none in No. 1); enzyme solution, 0·1 ml. (none in no. 2); water to 0·5 ml. total volume. Details of chromatography are given in the text.

Description	Period of incubation (min.)	Counts at origin	Counts in Pi spot	Total
Without $PM^{32}P$	5	—	—	—
Without enzyme	5	483	10	493
Complete mixture	0·7	452	38	490
Complete mixture	5	320	185	505
Complete mixture	60	12	447	459

There was no evidence of any other compounds on the chromatogram except PMP and Pi. The spots were counted on the paper with an end-window counter tube and the results, summarized in Table 8, confirm that no intermediates of low molecular weight other than orthophosphate were formed during the enzymic hydrolysis of PMP.

DISCUSSION

Three types of enzymes which break down metaphosphate have previously been described. The first type consists of a number of metaphosphatases isolated from various sources, all of which are hydrolytic enzymes which attack only relatively short chain molecules of the size of trimetaphosphate. An example of this type described recently is the enzyme isolated from pea seedlings by Pierpoint (1957); we tested samples of the enzyme which this author kindly supplied and found that it did not attack either the bacterial PMP or the synthetic PMP. The second type of polymetaphosphatase attacks long-chain metaphosphates of the molecular size of those isolated from bacteria and moulds as well as the smaller metaphosphates like tri- and hexametaphosphate. Malmgren (1952), working with an enzyme of this type isolated from *Aspergillus niger*, found that no inorganic phosphate was formed and from the results of kinetic studies has suggested that it can be described as a depolymerase. The third type of metaphosphatase is a transferase described by Kornberg *et al.* (1956), which in the presence of an unidentified primer transfers terminal orthophosphate from ATP to form high molecular weight PMP. This transferring enzyme is reversible and will split PMP by transferring Pi to form ATP from ADP. The enzyme which we have isolated from *Corynebacterium xerosis* appears to differ from these three types of metaphosphatases in that it attacks long-chain metaphosphates, with the formation of inorganic orthophosphate; as far as could be detected there was no formation of intermediate short-chain molecules and the enzyme does not attack metaphosphates of shorter chain length than hexametaphosphate. When tested under the conditions described by Kornberg (1957) for transferase activity there was no transfer of phosphate from PMP to ADP or AMP, neither did the addition of ATP or ADP affect the rate of enzyme reaction. The enzyme from *C. xerosis* thus appears to be a hydrolytic enzyme.

The significance of the enzymes which break down metaphosphate in micro-organisms must be related to the role which metaphosphate plays in the economy of the organism. Two different views have been expressed recently about this. Russian workers (Belozersky, 1958; Kulaev & Belozersky, 1957) regard the metaphosphates, because they are energy-rich compounds, as energy stores which can phosphorylate other compounds without the usual intervention of ATP or other nucleotides. In support of this idea it is claimed that metaphosphates are in combination with ribonucleoproteins, and together are concerned with nucleic acid and protein synthesis. In view of the rather drastic isolation procedures involved in the separation of the PMP complexes it is doubtful whether they exist as such in the cell. An alternative suggestion is that PMP may be regarded simply as a source of phosphate. This conception fits with the finding of many laboratories that polymetaphosphates accumulate when cells are given ample phosphate and energy supplies but growth retarded either by nitrogen or other starvation (Winder & Denneny, 1954; Sall, Mudd & Davis, 1956). Metaphosphate rapidly disappears from cells once normal growth is started after a period of starvation and the phosphate from PMP appears rapidly in most phosphate fractions of the cell (Mudd *et al.* 1958; Kulaev & Belozersky, 1957; Winder & Denneny, 1957). If, therefore, metaphosphate is regarded as a phosphate store the various metaphosphatases would presumably serve to break down the polymers to orthophosphate.

In connexion with the distribution of the various types of metaphosphatases in micro-organisms it is of significance that we have found an enzyme which splits the polymers to orthophosphate in *Corynebacterium xerosis* and which does not attack the short-chain molecules, since we found no short-chain metaphosphates to be present in the organism. In moulds and yeasts in which the depolymerase type of enzyme occurs, not only do the shorter chain compounds occur but enzymes which split them to orthophosphate have also been isolated.

Activation by EDTA and Mg^{++}. In the early experiments Mg^{++} was added to the enzyme assays because of the general finding that Mg^{++} stimulates this type of enzymes (Mattenheimer, 1951; Kornberg, 1957), and a small (20 %) stimulation was found with crude concentrated enzyme preparations; in higher concentrations however, Mg^{++} inhibited even in these crude preparations. With the purified enzyme, Mg^{++} did not stimulate and a higher concentration of Mg^{++} was markedly inhibitory. All the other metal ions tried were inhibitory. The addition of EDTA to the purified preparation stimulated in low concentration and inhibited in higher concentrations. A mixture of divalent metals did decrease the inhibitory effect of high concentration of EDTA, probably removing its effect by chelation. The inhibitory effect of Mg^{++} and divalent metals might be explained by their combination with the PMP in competition with the enzyme because PMP is a powerful chelating agent. The inhibition by high concentration of EDTA is possibly due to the chelation of activating metal bound to the enzyme.

REFERENCES

Belozersky, A. N. (1958). Polyphosphates, their formation, and importance for the processes of development of some lower organisms. *Proc. IV int. Congr. Biochem. Vienna* (in the Press).

Belozersky, A. N. & Kulaev, I. S. (1957). The polyphosphates and their significance in the development of *A. niger*. *Biokhimiya*, **22**, 29.

Berenblum, I. & Chain, E. (1938). An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295.

Berg, G. G. (1958). Ascending chromatography of polyphosphates. *Analyt. Chem.* **30**, 213.

Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). Determination of the serum proteins by means of biuret reaction. *J. biol. Chem.* **177**, 751.

Hanes, C. S. & Isherwood, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107.

Hughes, D. E. (1951). A press for disrupting bacteria and other micro-organisms. *Brit. J. exp. Path.* **32**, 97.

Juni, E., Kamen, M. D., Reiner, J. M. & Speigelman, S. (1948). Turnover and distribution of phosphorus compounds in yeast metabolism. *Arch. Biochem. Biophys.* **18**, 387.

Kalckar, H. M. (1947). Differential spectrophotometry of purine compounds by means of specific enzymes. III. Study of the purine metabolism. *J. biol. Chem.* **167**, 461.

Katchman, B. J. & Van Wazer, J. R. (1954). The soluble and insoluble polyphosphates of yeast. *Biochim. Biophys. Acta*, **14**, 445.

Kitasato, T. (1928). Über Metaphosphatase. *Biochem. Z.* **197**, 257.

Kornberg, A., Kornberg, S. R. & Simms, E. S. (1956). Metaphosphate synthesis by an enzyme from *E. coli*. *Biochim. Biophys. Acta*, **20**, 215.

Kornberg, S. R. (1957). Adenosine triphosphate synthesis from polyphosphate by an enzyme from *Escherichia coli*. *Biochim. Biophys. Acta*, **26**, 294.

Krishnan, P. S., Damle, S. P. & Bajaj, V. (1957). Formation of soluble and insoluble metaphosphates in *A. niger*. *Arch. Biochem.* **67**, 35.

Kulaev, I. S. & Belozersky, A. N. (1957). A study of the physiological role of polyphosphates in the development of *A. niger* using radio-phosphorus ^{32}P. *Biokhimiya*, **22**, 587.

Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658.

Mackie, T. J. & McCartney, J. E. (1945). *Handbook of Practical Bacteriology*. Edinburgh: E. and S. Livingstone Ltd.

Malmgren, H. (1952). Enzymatic breakdown of polymetaphosphate: purification and specificity of the enzyme. *Acta chem. scand.* **6**, 16.

Mann, T. (1944). Isolation of pyrophosphate and metaphosphate from *A. niger*. *Biochem. J.* **38**, 345.

Mattenheimer, H. (1951). Die enzymatische Aufspaltung anorganischer Poly und Metaphosphate durch organextrakte und Trockenhefe. *Biochem. Z.* **322**, 36.

Mudd, S., Yoshida, A. & Koike, M. (1958). Polyphosphate as accumulator of phosphate and energy. *J. Bact.* **75**, 224.

Pfanstiel, R. & Iler, R. K. (1952). Potassium metaphosphate: molecular weight, viscosity behaviour and rate of hydrolysis of non cross-linked polymer. *J. Amer. chem. Soc.* **74**, 6059.

Pierpoint, W. S. (1957). Phosphatase and metaphosphatase activities of pea extracts. *Biochem. J.* **65**, 67.

Potter, V. R. & Elvehjem, C. A. (1936). A modified method for the study of tissue oxidations. *J. biol. Chem.* **114**, 495.

Sall, T., Mudd, S. & Davis, J. C. (1956). Factors conditioning the appearance and disappearance of metaphosphate in cells of *Corynebacterium diphtheria*. *Arch. Biochem.* **60**, 130.

Wiame, J. M. (1949). The occurrence and physiological behaviour of two metaphosphate fractions of yeast. *J. biol. Chem.* **178**, 919.

Winder, F. G. & Denneny, J. M. (1954). Phosphorus metabolism of mycobacteria: determination of phosphorus compounds in some mycobacteria. *J. gen. Microbiol.* **15**, 1.

Winder, F. G. & Denneny, J. M. (1957). The metabolism of inorganic polyphosphates in mycobacteria. *J. gen. Microbiol.* **17**, 573.

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Edwards, M. R., Hazen, E. L. & Edwards, G. A. (1959). *J. gen. Microbiol.* **20**, 496–503

The Fine Structure of the Yeast-like Cells of Histoplasma in Culture

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SUMMARY: The fine structure of the yeast-like cells of *Histoplasma capsulatum* Darling and *H. duboisii* Vanbreuseghem has been studied and compared by electron microscopy. The micro-morphology of single and budding cells, as well as some details of the budding process, are described. No evidence of a capsule or slime layer on the outside of the cell wall of either species was demonstrated. In both species the cell walls were found to be laminated and in general slightly thicker in *H. duboisii* than in *H. capsulatum*. The possibility of the laminations being associated with the presence of chitin in the framework of the cell walls is discussed. The cells of the two species, strikingly similar in structure, were found to be highly differentiated, resembling animal cells in some of their micromorphological features, namely in the presence of endomembrane systems, in mitochondrial numbers and forms, and in nuclear fine structure.

Since the discovery by Darling (1906) of *Histoplasma capsulatum*, this fungus has been the subject of considerable cytological and histochemical study (Rocha-Lima, 1913; De Monbreun, 1934; Ciferri & Redaelli, 1934; Conant, 1941; Dowding, 1948; Kligman & Baldridge, 1951; Milne, 1957). These contributions have elucidated some morphological features of the fungus, such as the presence of a cell wall and the nucleus of the yeast-like cells, as well as the structure of the hyphae and the different types of spores developed in the saprophytic growth cycle. However, due to the small size (1–5 μ) of the yeast-like cells of this species, in the parasitic form as well as in culture, it is not possible with either the ordinary light microscope or the phase-contrast microscope to obtain a precise picture of the cellular components and it has been even more difficult to determine whether or not the cell is surrounded by a thin capsule or slime layer.

Recently, a different type of *Histoplasma* has been described in cases of tropical-African origin (Catanei & Kervran, 1945; Duncan, 1947; Dubois, Janssens & Brutsaert, 1952; Dubois & Vanbreuseghem, 1952; Vanbreuseghem, 1953), distinguishable from the classical type by its large intracellular parasitic form, which is by linear measurement three to six times the size of *H. capsulatum*. It was named *Histoplasma duboisii* by Vanbreuseghem (in Dubois, Janssens & Brutsaert, 1952) but its status as a species is still controversial. Comparative studies of both forms of *Histoplasma* have appeared in the literature (Drouhet & Schwarz, 1956*a*, *b*; Mariat & Segretain, 1956; Vanbreuseghem, 1956; Drouhet, 1957; Schwarz & Drouhet, 1957; Schwarz, 1958*a*), and more recently Schwarz and Emmons (Schwarz, 1958*b*) have intimated acceptance of the new species.

Notwithstanding the differences in size and in tissue reaction of the parasitic forms (Edwards, Edwards & Hazen, 1959), the two types of *Histoplasma* seem to be indistinguishable in culture. The mycelial phase shows the same morphological characteristics and the yeast phase consists principally of the classical small, thin-walled, ovoid, yeast-like cells (1–5μ in length), among which a variable number of larger, thick-walled, budding cells (over 5μ in length) are found. A good description of the latter was given by Duncan (1958), who also noted that *H. duboisii* seemed to have a more marked tendency than *H. capsulatum* to develop the large cells in response to unfavourable conditions.

This paper presents the results of a comparative cytological investigation, using the electron microscope and thin sectioning techniques, of the two types (or species) of *Histoplasma* in the yeast phase.

METHODS

Four strains of *Histoplasma* in the yeast phase were employed, two of *H. capsulatum* Darling (nos. 4890 and 5854), and two of *H. duboisii* Vanbreuseghem (nos. 58136 and 58134). Strain no. 4890 was obtained from the late Dr Rhoda Benham of Columbia University, New York, New York, and no. 5854 was isolated in 1957 in this laboratory from sputum from a patient with pulmonary histoplasmosis. Strains nos. 58136 and 58134 were received under nos. RV 7408 and RV 4910, respectively, through the courtesy of Dr R. Vanbreuseghem of the Institut de Médecin Tropicale, Antwerp, Belgium. They were in the mycelial phase when received and were converted to the yeast phase by passage through mice.

The yeast-phase cultures were obtained from solid and liquid media. Francis's glucose-cystine blood agar slants (Campbell, 1947), and a defined liquid medium (Salvin, 1950) dispensed in 125 ml. Erlenmeyer flasks in 50 ml. amounts were used. The cultures on agar slants were sealed with paraffin and incubated at 37° for 48–72 hr.; the broth cultures were shaken manually twice each day for about 3 min., during the incubation period of 48–72 hr. at 37°.

The fixation and embedding procedures were as follows:

(A) From the agar slant cultures, the growth was transferred directly by means of a nichrome wire loop to a 2 % (w/v) OsO_4 solution in veronal + alacetate buffer at pH 7·4; or the growth was washed off in a physiological salt solution, centrifuged and then suspended in a small volume of the veronal + acetate buffer (pH 7·4) to which an equal volume of 3 % (w/v) OsO_4 was added, so as to have a final concentration of 1·5 % OsO_4.

(B) The broth cultures were centrifuged in large tubes for 15–20 min. at about 1900 rev./min., and the sediment suspended in 3–5 ml. of veronal + acetate buffer solution (pH 7·4) and mixed with an equal volume of a cold 3 % (w/v) OsO_4 solution in the same buffer. The final mixture (1·5 % OsO_4) was kept at 4–5° during 20–30 min. Thereafter, the fixation proceeded at room temperature.

After 1 hr. this mixture was centrifuged as indicated above, and the sediment resuspended in 3 ml. of a buffered 1·5 % OsO_4 solution (pH 7·4).

In all instances, the last step of fixation was accomplished in a small tube suitable for the Servall centrifuge, using about 5 ml. of the fixing fluid, the fixation time varying from 1 to 3 hr. Fixation of 1 hr. and 40 min. in 2 % OsO_4, and 2 hr. in 1·5 % OsO_4 gave the best results. After fixation, the commonly used schedule of washing in buffer and dehydrating in graded aqueous ethanol was followed. Before each change of solution, the material was centrifuged at low speed to a compact pellet and between changes the cells were agitated gently by hand.

Embedding was done in a mixture of 90 % butyl methacrylate and 10 % methyl methacrylate, with 1·5 % of Lucidol as the initiator, at 48° for 36–40 hr.

Sections were cut with a diamond knife in the Porter–Blum microtome. They were supported on formvar lightly stabilized by carbon, and observed in RCA and Siemens electron microscopes at initial magnifications of × 2600 to × 20,000.

RESULTS

The cells on either solid or liquid media were similar in structure, hence they will be considered together.

Single yeast-like cells

Histoplasma capsulatum. The yeast-like cells of *H. capsulatum* are generally oval to spherical in outline. Some irregular forms have been observed, but it is believed that such irregularities result from fixation and embedding procedures. The measurements of 116 cells averaged 1·73 μ by 2·47 μ in their short and long axes respectively, with variations from 0·53–4·60 μ in the long axis. The cell is delimited by a laminated cell wall of an average thickness of 90 mμ which appears to be smooth in outline. The outer limit of the cell wall is a thin layer of high electron-density (Pl. 1, figs. 1, 2) averaging 16 mμ in width, accompanied by inner layers of medium density. The innermost layer is intimately apposed or adherent to the plasma membrane, being evident only when the cytoplasm is pulled away from the cell wall as in plasmolized or degenerated cells. It then appears similar in density and thickness to the outer layer described above.

The plasma membrane (or cytoplasmic membrane—O'Hern & Henry, 1956; Agar & Douglas, 1957) is of high electron density and averages 8 mμ in thickness (Pl. 1, fig. 1; Pl. 2, figs. 4, 6). In few cells is the plasma membrane regular. More commonly it is extremely rugose or serrate and exhibits considerable vesiculation, indicative of pinocytotic activity.

The nucleus occupies a large portion of the cell and is usually ovoid, at times irregular; it averages 1·08 μ by 1·63 μ in short and long axes. The nucleus possesses one or more nucleoli which have a generally homogeneous matrix of granules of high density (Pl. 1, figs. 1, 2) and which may be fragmented or vacuolated. The chromatic material appears mostly scattered throughout the matrix. The nucleus is bounded by a thin membrane of 10 mμ thickness which in turn is surrounded by a fairly regular perinuclear cisterna of an average width of 28 mμ. The outer envelope of the nucleus averages 10 mμ in thickness and at times is seen to be continuous with membranes of the endoplasmic reticulum (Pl. 2, fig. 3). Pores are found at irregular intervals along the nuclear border (Pl. 3, fig. 8), oftentimes in front of mitochondria which are adjacent to the nucleus. The outer nuclear membrane may be continuous with the outer membrane of a neighbouring mitochondrion.

The cytoplasm of the cell contains tubules and vesicles of the endoplasmic reticulum (Pl. 1, fig. 2; Pl. 5, fig. 16), scattered and clumped granules, lipid bodies and mitochondria. In some cells, there appear to be vesicles and lamellae, suggestive of the reticular apparatus of Golgi, apparently associated with the presence of lipid (Pl. 1, fig. 2; Pl. 3, fig. 7). The form of this organelle does not appear as distinct as it usually does in higher cells. The mitochondria are abundant, in general 4–5 per cell, usually oval in outline, averaging 470 mμ by 660 mμ. They contain relatively few cristae mitochondriales and generally have a matrix of fine granules or filaments of low density, thus giving the appearance of being virtually empty (Pl. 1, fig. 2). The lipid bodies (Pl. 1, fig. 2) are common, usually 2 per cell (Pl. 4, fig. 12). They are, in general, of regular outline, oftentimes limited by a membrane, but in some instances they appear as scattered irregular masses.

Histoplasma duboisii. The single yeast cells of *H. duboisii* are generally similar to those of *H. capsulatum.* They possess the same nuclear and cytoplasmic components and are of the same shape (Pl. 2, figs. 5, 6). *H. duboisii* cells average 1·92 μ by 2·75 μ in their short and long axes, respectively, with variations from 0·77 to 5·86 μ in the longer axis. The cell wall is laminated, averaging 100 mμ in thickness, having an outer and denser layer of an average of 10 mμ and an innermost layer of the same thickness. The plasma membrane usually exhibits considerable pinocytotic activity and averages 9 mμ in thickness.

The *Histoplasma duboisii* cells, observed under the present conditions, appear to be of two types or stages, differing in the density or packing of the cytoplasmic and nuclear components: (*a*) light cells which show a virtually empty cytoplasm (Pl. 2, fig. 6), i.e. the constituent granules are clumped, as previously observed in *H. capsulatum*; (*b*) dark cells (Pl. 2, fig. 5) which possess a closely packed, finely granular cytoplasmic matrix and a nuclear matrix, fuller and more finely granular in nature than the light cells. The other cellular components are similar to those of *H. capsulatum*, and need not be described further.

Budding yeast-like cells

Histoplasma capsulatum. Budding cells of *H. capsulatum* were observed in various stages in cultures of 48–72 hr. The earliest indication of a budding process seems to be a thickening of the cell wall around a small nipple-like evagination of the cytoplasm (Pl. 3, fig. 10). The plasma membrane in the evaginating area may show extreme vesiculation. The evagination continues until a small thumb-like process, containing only cytoplasmic matrix, is formed (Pl. 4, fig. 11). The head of this process then enlarges and becomes distinguishable from the neck but still contains only cytoplasmic matrix. In the next apparent stage, membranes may be seen in the neck and in the head of the new formation (Pl. 4, fig. 12), with the nucleus of the mother cell appearing in the area nearest the neck of the bud (Pl. 4, fig. 13). In older buds mitochondria appear (Pl. 4, fig. 12). In an apparently later stage, nuclear material is seen in the parent cell, in the neck, and also in the proximal portion of the bud (Pl. 4, fig. 13). This is better illustrated in *H. duboisii* (Pl. 6, fig. 18). In a more advanced step a definitive nucleus appears in the bud itself (Pl. 4, fig. 14; Pl. 5, figs. 15, 16). (Since this study was completed, Hashimoto, Conti & Naylor, 1958, have well shown the nuclear migration and vegetative division in bud-formation in *Saccharomyces*.) The plasma membrane of the bud shows the same vesiculation as that of the mother cell (Pl. 5, fig. 16). After the appearance of the definitive nucleus in the bud, the neck becomes constricted and while still maintaining this narrow connexion, the bud enlarges in size, approximating the size of the parent cell, and acquires the mature complement of cytoplasmic components, i.e. numerous mitochondria, endoplasmic reticulum, lipid granules and Golgi apparatus (Pl. 4, fig. 14; Pl. 5, figs. 15, 16). The connexion between mother and daughter cells becomes quite tenuous, and eventually their respective cytoplasms are separated by their plasma membranes. This step is followed by an invasion, between the two cells, of less dense, laminar material of the still continuous cell wall. Secondarily, a dense line, normal to the long axes of the cells, appears in the new cell wall between mother and daughter cells (Pl. 5, fig. 15). As an apparent last step the two cells separate entirely by the splitting of the cell wall material along the dense line (or splitting plate) just described and the bud becomes a complete cell.

Histoplasma duboisii. The budding process is similar to that described for *H. capsulatum.* A few micrographs of budding cells of *H. duboisii* are presented (Pl. 6, figs. 17–19) to illustrate this similarity. There is, however, a difference in electron density between the mother and the daughter cells of *H. duboisii.* The cytoplasmic and nuclear matrices of the bud are of higher density and therefore appear darker in the micrograph, so that its fine structure is difficult to observe (Pl. 6, fig. 19). This finding suggests that the distinction between the light and dark cells may be associated with differences in age and metabolic activity.

DISCUSSION

A capsule, or thin layer of slime, is not present on the outside of the cell of either species of *Histoplasma.* These findings are in agreement with those of Kligman & Baldridge (1951), Salvin & Ribi (1955) and Ribi & Salvin (1956) who used different electron microscopic techniques. It could perhaps be argued that the procedure of fixing and dehydrating washed off the capsular material from the cells. Such criticism, however, would not seem justified in view of the fact that other micro-organisms such as *Cryptococcus neoformans* (unpublished data) and *Chlamydomonas pyriforme* (Sager & Palade, 1957), known to possess a capsule, when studied by the same technique were not completely deprived of their capsular material.

In both species of *Histoplasma* the cell walls are laminated, appearing to be comparable in density and structure to the basement membrane of some insect cells (cf. Pl. 134, fig. 4, in Edwards, Ruska & de Harven, 1958). It seems of interest to mention here that the basement membrane of insect cells is composed of chitin and protein, and that the framework of the fungi causing systemic diseases has also been found to be composed of chitin (Blank, 1954). A similar laminated cell wall has been described in *Saccharomyces cerevisiae* in which chitin was also mentioned as present (Agar & Douglas, 1955). The correlation between the laminated structure of these cell walls and their chitinous framework, however, remains to be proven.

The comparative cytological investigation of the two species of *Histoplasma* in culture revealed no significant differences. The size of the cells of *H. duboisii* was very close to that of *H. capsulatum*, and in both species the nucleus and the cytoplasmic organelles showed the same fine structure. However, the younger cells of *H. duboisii* were generally denser to the electron beam than the older cells, a distinction not found in *H. capsulatum*, under the conditions of this study. This difference alone would not seem to be of significance. A much more striking distinction was found in the study of the two species in mouse spleen (Edwards, Edwards & Hazen, 1959), in which the cells of *H. duboisii* were, on the average, 5·5 times larger than the cells of *H. capsulatum*.

REFERENCES

Agar, H. D. & Douglas, H. C. (1955). Studies of budding and cell wall structure of yeast. *J. Bact.* **70**, 427.

Agar, H. D. & Douglas, H. C. (1957). Studies on the cytological structure of yeast: electron microscopy of thin sections. *J. Bact.* **73**, 365.

Blank, F. (1954). On the cell walls of dimorphic fungi causing systemic infections. *Canad. J. Microbiol.* **1**, 1.

Campbell, C. C. (1947). Reverting *Histoplasma capsulatum* to the yeast phase. *J. Bact.* **54**, 263.

Catanei, A. & Kervran, P. (1945). Nouvelle mycose humaine observée au Soudan Français. *Arch. Inst. Pasteur Algér.* **23**, 169.

Ciferri, R. & Redaelli, P. (1934). *Histoplasma capsulatum* Darling, the agent of 'histoplasmosis': systematic position and characteristics. *J. trop. Med.* (*Hyg.*), **37**, 278.

Conant, N. F. (1941). A cultural study of the life cycle of *Histoplasma capsulatum* Darling 1906. *J. Bact.* **41**, 563.

Darling, S. T. (1906). A protozoan general infection producing pseudo-tubercles in the lungs and focal necrosis in the liver, spleen and lymph nodes. *J. Amer. med. Ass.* **46**, 1283.

DeMonbreun, W. A. (1934). The cultivation and cultural characteristics of Darling's *Histoplasma capsulatum*. *Amer. J. trop. Med.* **14**, 93.

Dowding, E. S. (1948). The spores of *Histoplasma*. *Canad. J. Res.* E, **26**, 265.

Drouhet, E. (1957). Quelques aspects biologiques et mycologiques de l'histoplasmose. *Arch. biol. Med.* **33**, 439.

Drouhet, E. & Schwarz, J. (1956*a*). Comparative studies with 18 strains of *Histoplasma*. Morphology in tissues and virulence of African and American strains. *J. Lab. clin. Med.* **47**, 128.

Drouhet, E. & Schwarz, J. (1956*b*). Croissance et morphogenese d'*Histoplasma*. I. Etude comparative des phases mycélienne et levure de 18 souches d'*Histoplasma capsulatum* d'origine americaine et africaine. *Ann. Inst. Pasteur*, **90**, 144.

Dubois, A., Janssens, P. G. & Brutsaert, P. (1952). Un cas d'histoplasmose africaine, avec une note mycologique sur *Histoplasma duboisii* n.sp., par R. Vanbreuseghem. *Ann. Soc. belge Med. trop.* **32**, 569.

Dubois, A. & Vanbreuseghem, R. (1952). L'histoplasmose africaine. *Bull. Acad. Med. Belg.* **17**, 551.

Duncan, J. T. (1947). A unique form of *Histoplasma. Trans. Roy. Soc. trop. Med. Hyg.* **40**, 364.

Duncan, J. T. (1958). Tropical African histoplasmosis. *Trans. Roy. Soc. trop. Med. Hyg.* **52**, 468.

Edwards, G. A., Edwards, M. R. & Hazen, E. L. (1959). An electron microscopic study of *Histoplasma* in mouse spleen. *J. Bact.* **77**, No. 4.

Edwards, G. A., Ruska, H. & de Harven, E. (1958). Neuromuscular junctions in flight and tymbal muscles of the cicada. *J. Biophys. Biochem. Cytol.* **4**, fig. 4.

Hashimoto, T., Conti, S. F. & Naylor, H. B. (1958). Nuclear changes occurring during bud-formation in *Saccharomyces cerevisiae* as revealed by ultra-thin sectioning. *Nature, Lond.* **182**, 454.

Kligman, M. & Baldridge, G. D. (1951). Morphology of *Sporotrichum schenckii* and *Histoplasma capsulatum* in tissue. *A.M.A. Arch. Path.* **51**, 567.

Mariat, F. & Segretain, G. (1956). Étude mycologique d'une histoplasmose spontanée du singe africain (*Cynocephalus babuin*). *Ann. Inst. Pasteur*, **91**, 874.

Milne, H. A. (1957). The morphology and cytochemistry of *H. capsulatum. J. Med. Lab. Tech.* **14**, 142.

O'Hern, E. M. & Henry, B. S. (1956). A cytological study of *Coccidioides immitis* by electron microscopy. *J. Bact.* **72**, 632.

Ribi, E. & Salvin, S. B. (1956). Antigens from the yeast phase of *Histoplasma capsulatum.* I. Morphology of the cell as revealed by the electron microscope. *Exp. Cell. Res.* **10**, 394.

Rocha-Lima, H. (1913). Beitrag zur Kenntnis der Blastomykosen Lymphangitis epizootica und Histoplasmosis. *Zbl. Bakt.* **67**, 233.

Sager, R. & Palade, G. E. (1957). Structure and development of the chloroplast in *Chlamydomonas.* I. The normal green cell. *J. Biophys. Biochem. Cytol.* **3**, 463.

Salvin, S. B. (1950). Growth of the yeastlike phase of *Histoplasma capsulatum* in a fluid medium. *J. Bact.* **59**, 312.

Salvin, S. B. & Ribi, E. (1955). Antigens from yeast phase of *Histoplasma capsulatum.* II. Immunologic properties of protoplasm vs. cell walls. (22012). *Proc. Soc. exp. Biol., N.Y.* **90**, 287.

Schwarz, J. (1958*a*). The pathogenesis of histoplasmosis. *Trans. N.Y. Acad. Sci.* **20**, 541.

Schwarz, J. (1958*b*). *Proceedings of the Histoplasmosis Seminar*, no. 12. Cincinnati, Ohio, The Jewish Hospital Association.

Schwarz, J. & Drouhet, E. (1957). Morphologic features of an African strain of *Histoplasma* in hamsters and mice. *A.M.A. Arch. Path.* **64**, 409.

Vanbreuseghem, R. (1953). *Histoplasma duboisii* and African histoplasmosis. *Mycologia*, **45**, 803.

Vanbreuseghem, R. (1956). *Histoplasma duboisii* and large forms of *Histoplasma capsulatum. Mycologia*, **48**, 264.

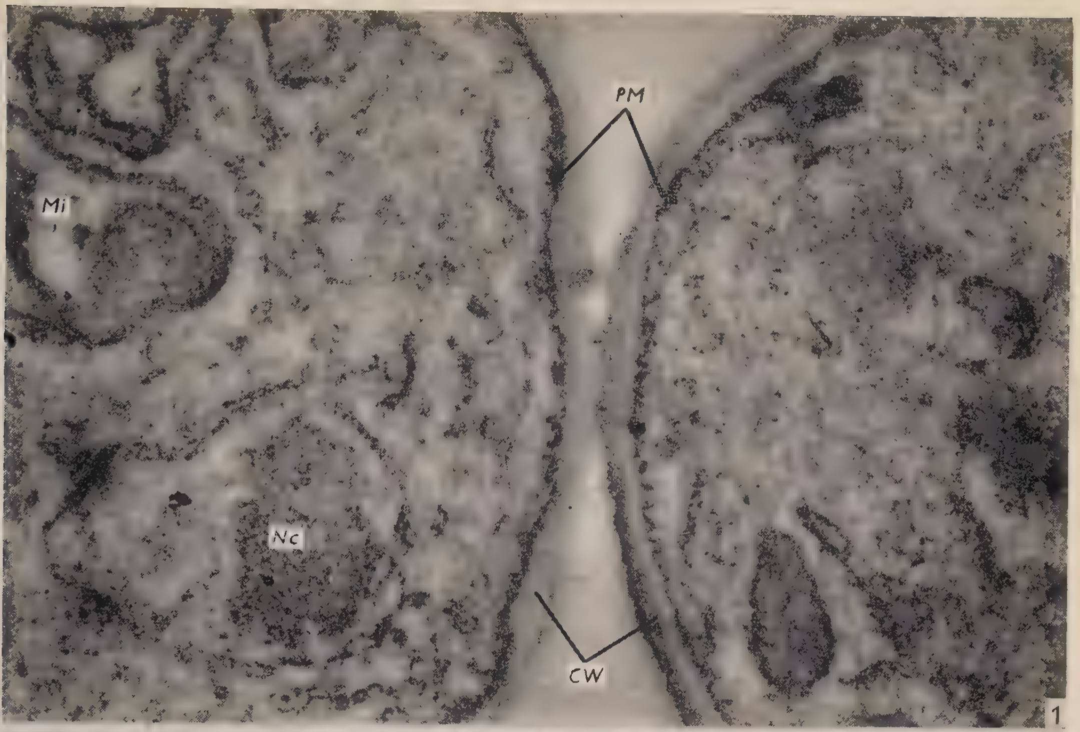
EXPLANATION OF PLATES

Symbols used in figures: *CW*, cell wall; *PM*, plasma membrane; *Nu*, nucleus; *Nc*, nucleolus; *Mi*, mitochondrion; *er*, endoplasmic reticulum; *G*, reticular apparatus of Golgi; *L*, lipid body.

Plate 1

Fig. 1. Portions of two cells of *Histoplasma capsulatum.* Each cell is bounded by a relatively thick, layered cell wall and a smooth to irregular plasma membrane. The cytoplasm contains scattered granules, membrane-bound profiles and relatively large mitochondria. The nucleolus of the left-hand cell displays a prominent nucleolus. × 50,000.

Fig. 2. *Histoplasma capsulatum.* The plasma membrane shows considerable pinocytosis. The nucleus has a dense nucleolus. Lipid bodies occur near the few membranes of the Golgi apparatus. The mitochondrion possesses few, incomplete cristae. Membrane bound profiles of the endoplasmic reticulum appear as vesicles or tubules. × 40,000.



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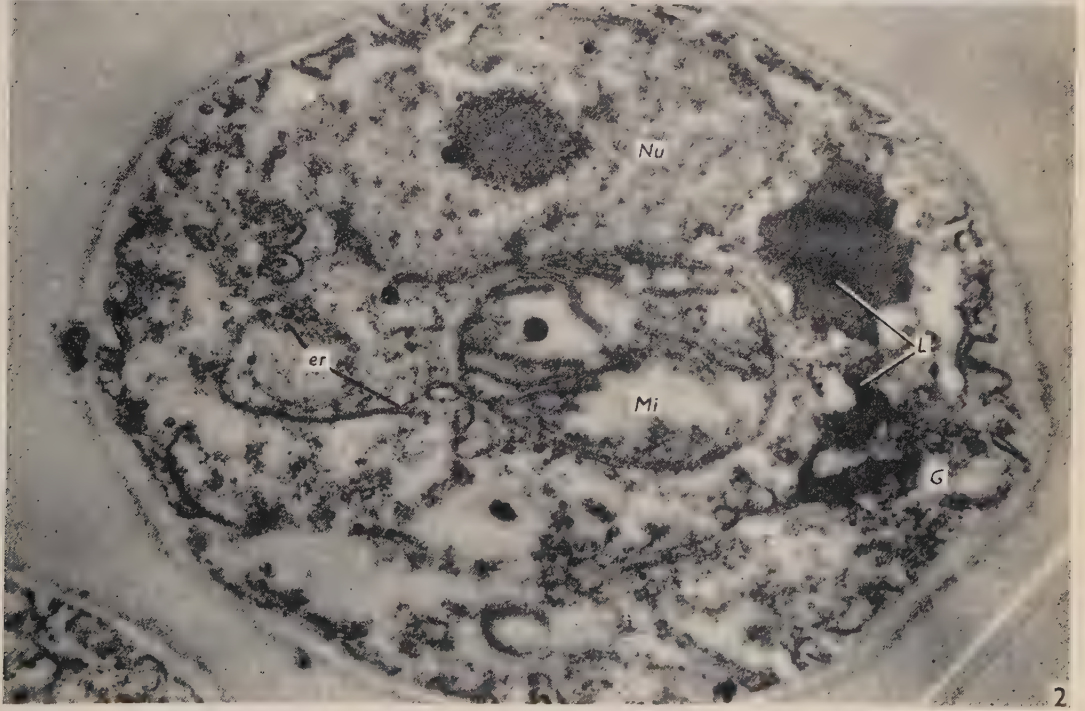

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M. R. Edwards, E. L. Hazen & G. A. Edwards—The fine structure of Histoplasma. Plate 1

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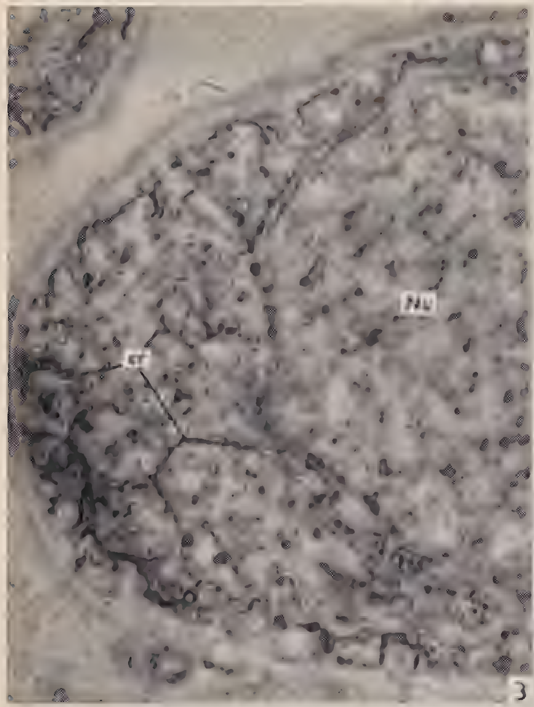

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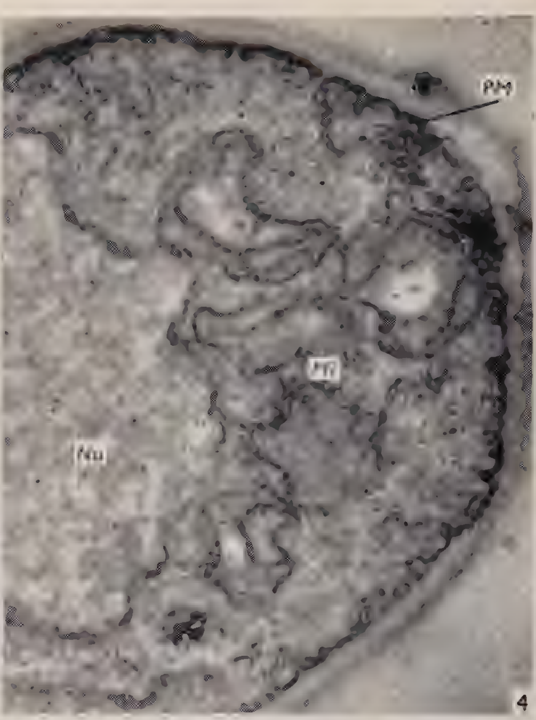

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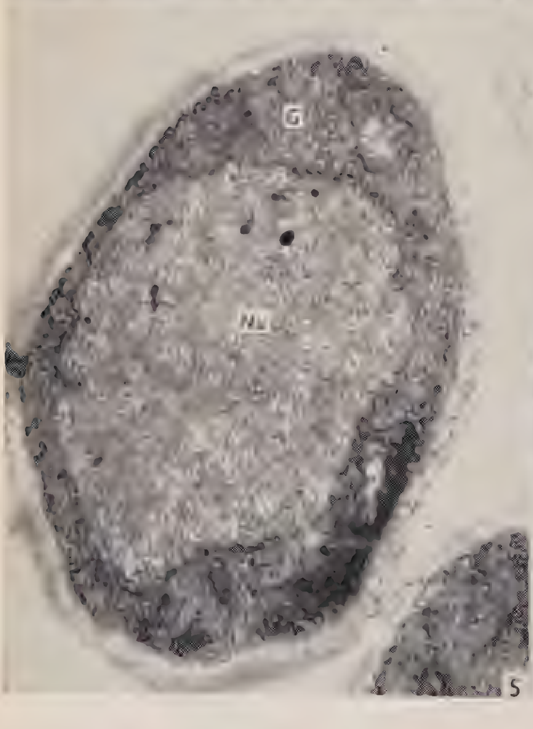

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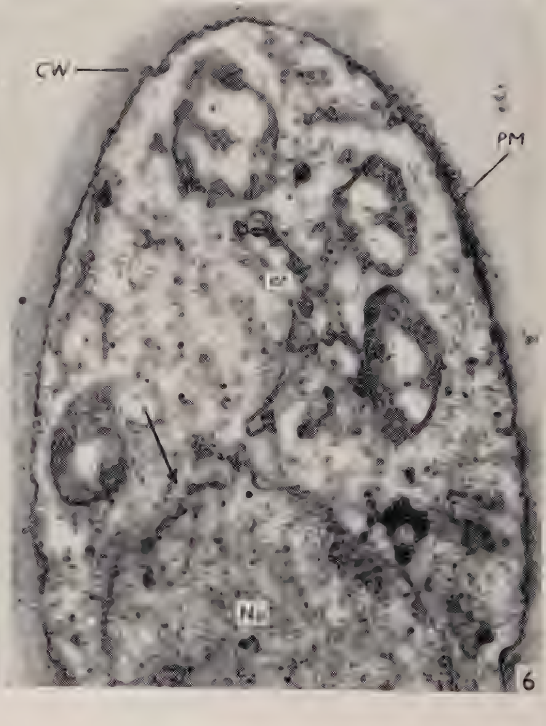

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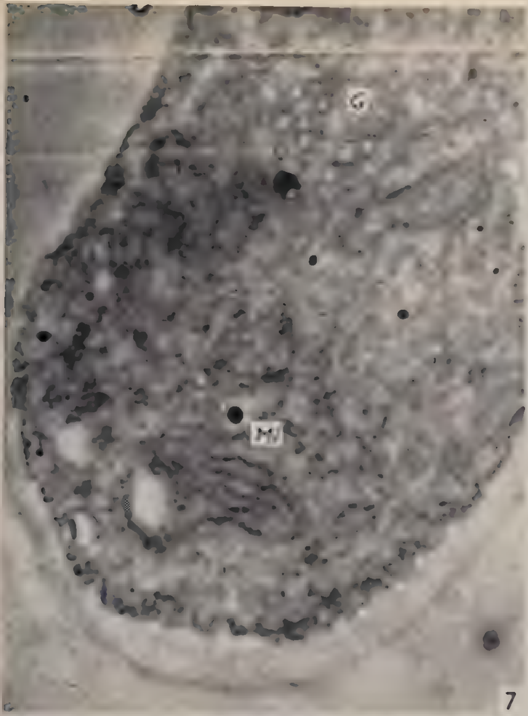
M. R. Edwards, E. L. Hazen & G. A. Edwards—The fine structure of Histoplasma. Plate 2



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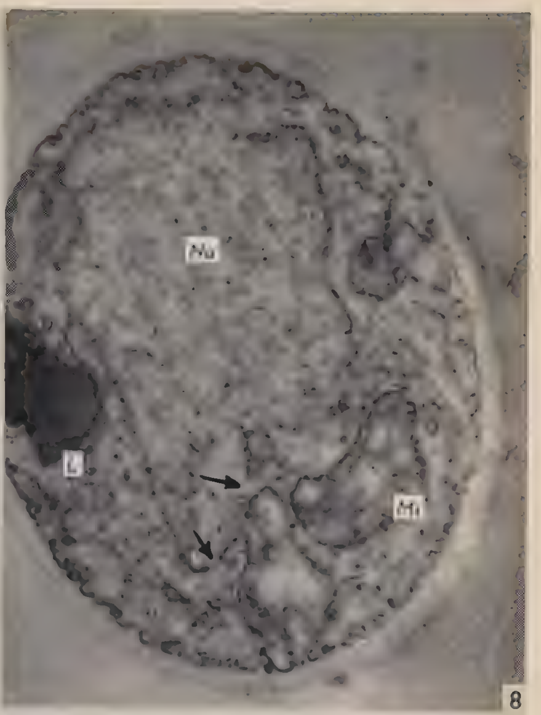

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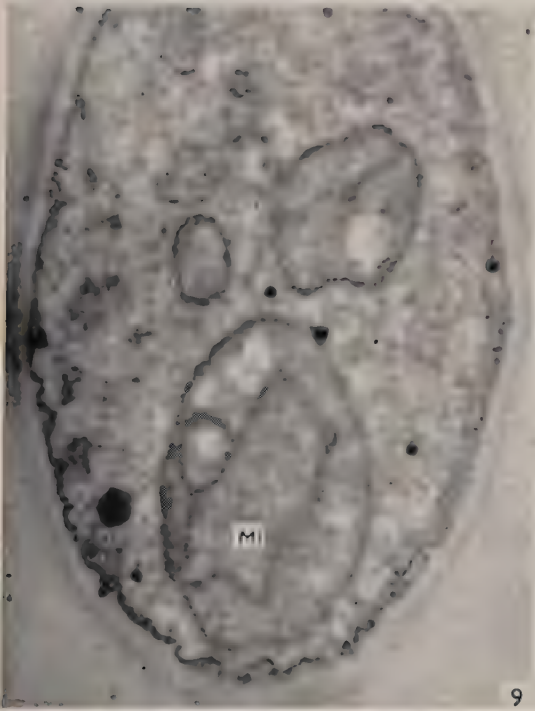

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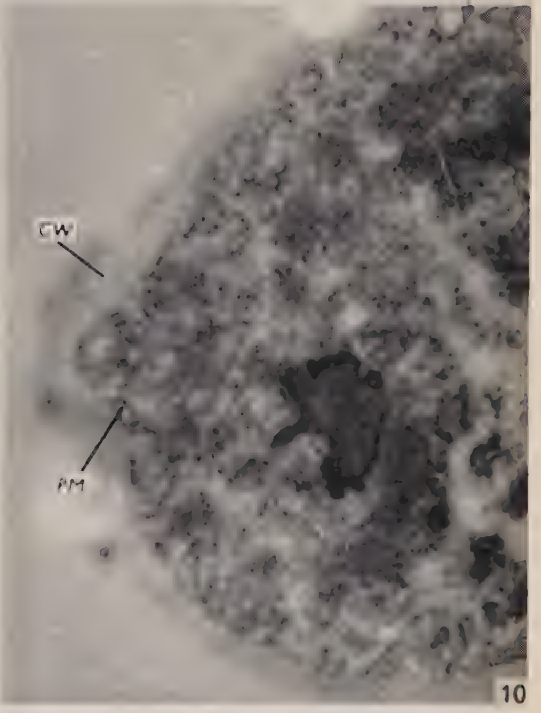

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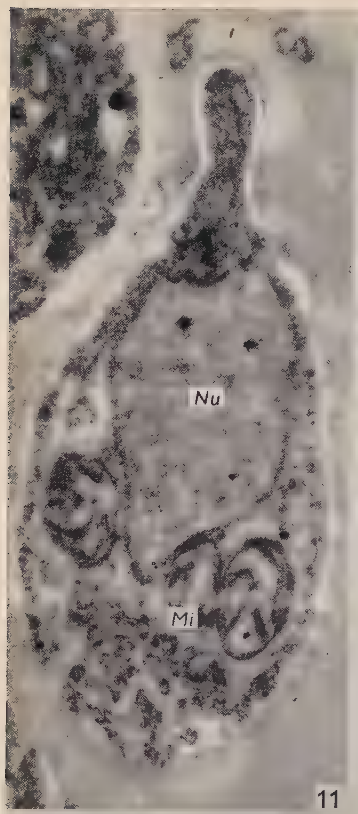
M. R. Edwards, E. L. Hazen & G. A. Edwards—The fine structure of Histoplasma. Plate 3



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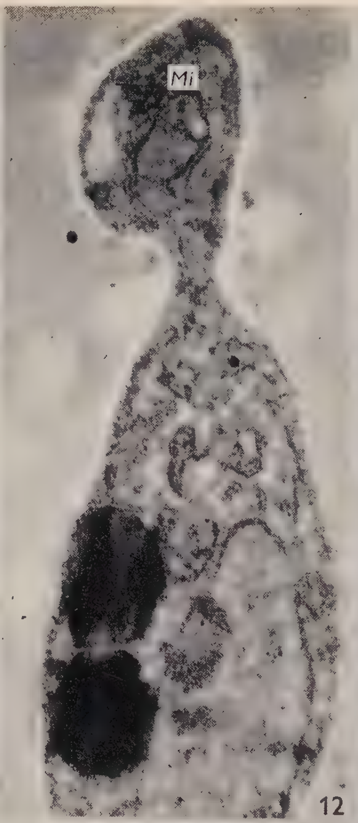

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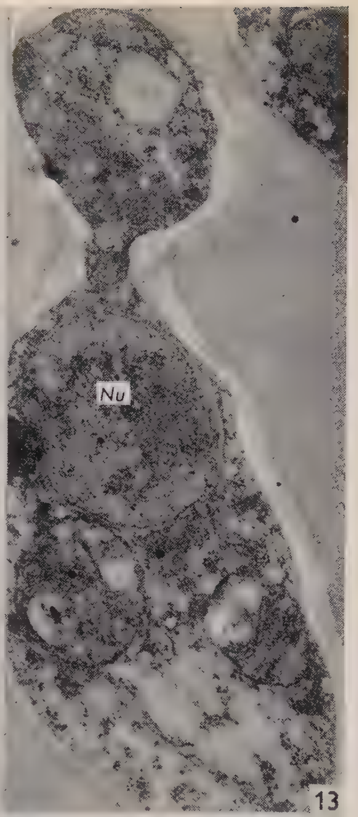

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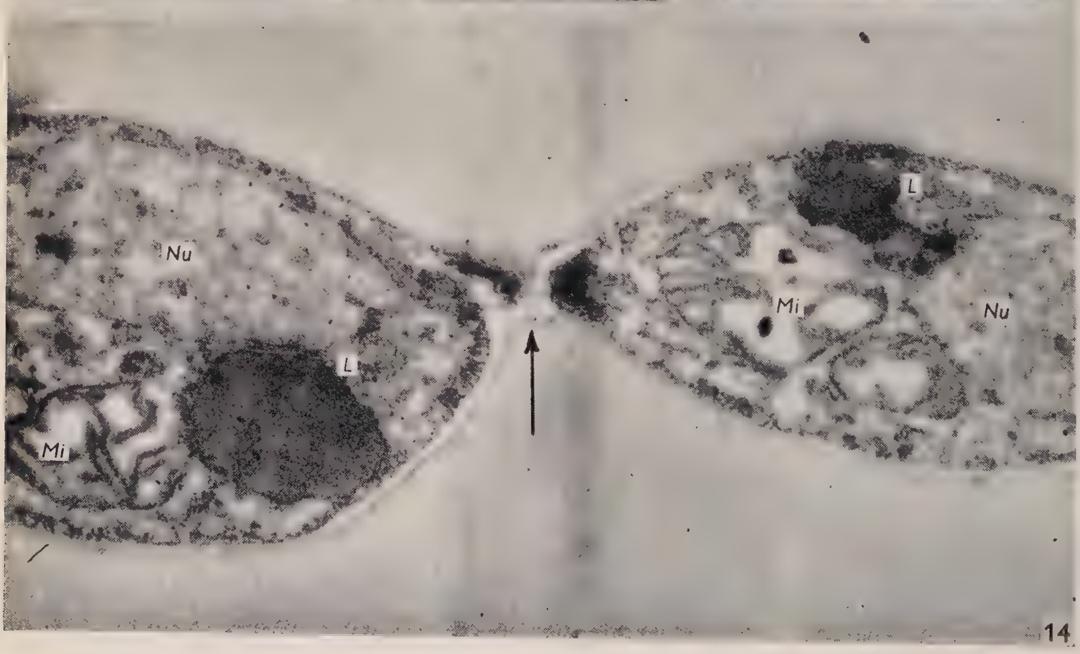
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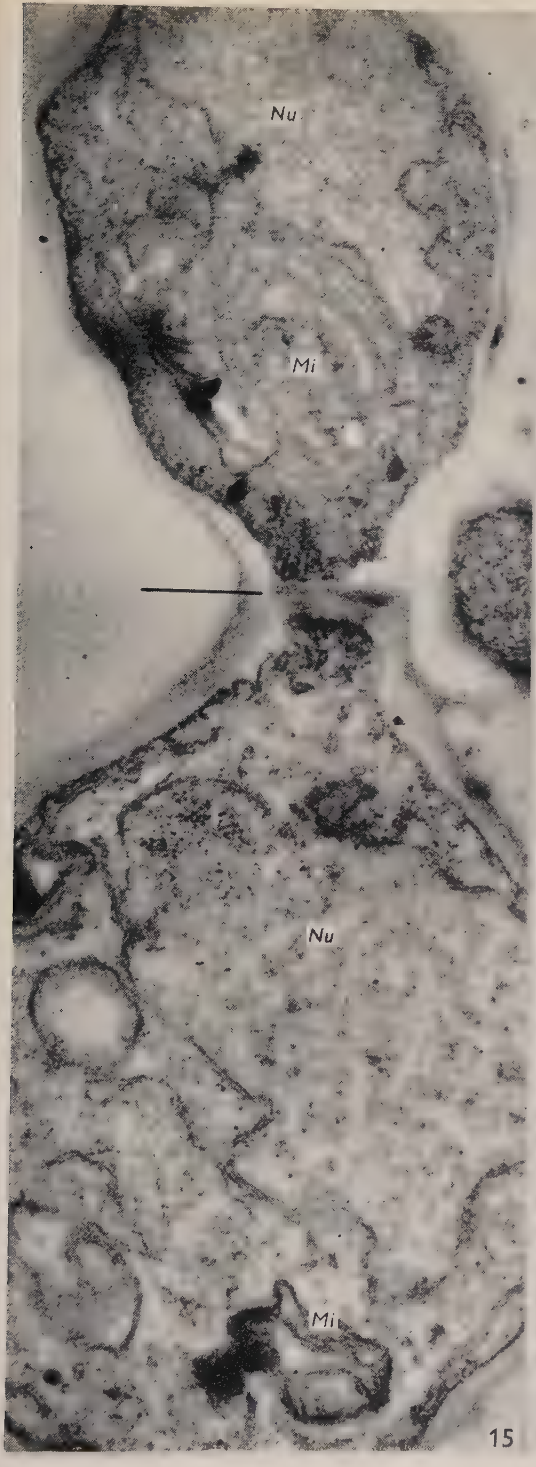
M. R. Edwards, E. L. Hazen & G. A. Edwards—The fine structure of Histoplasma. Plate 4



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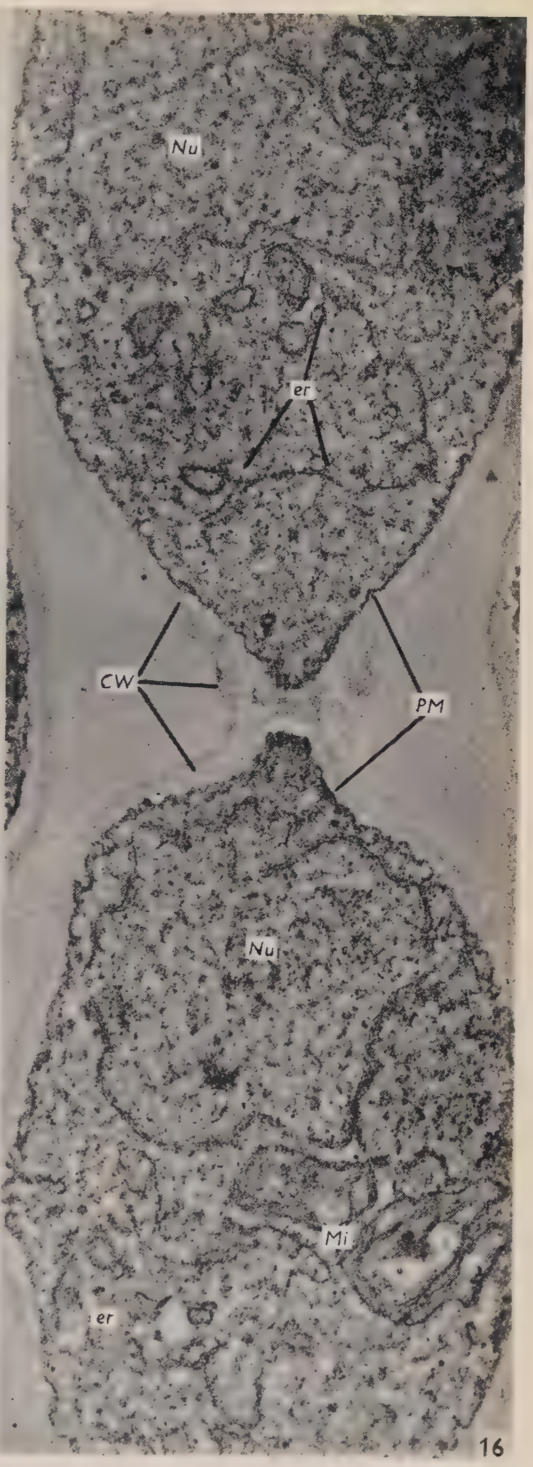

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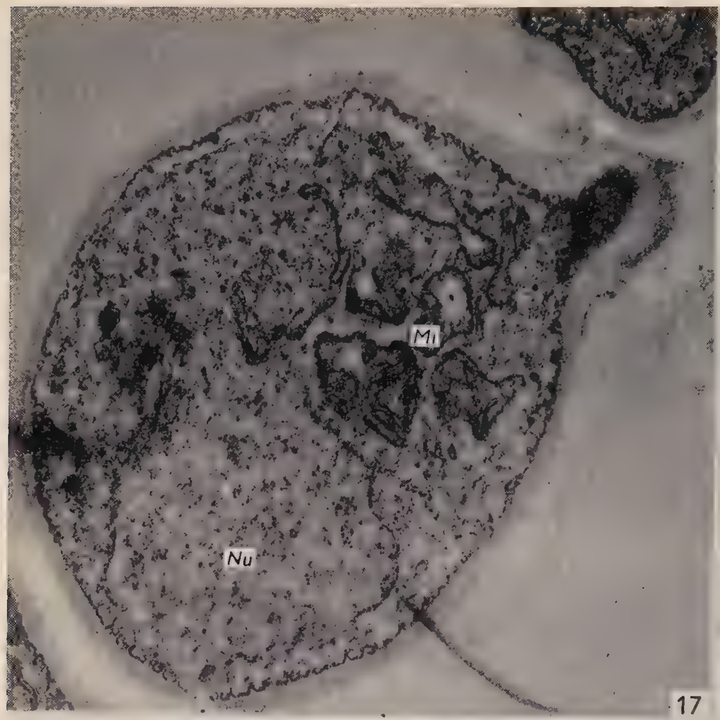
M. R. Edwards, E. L. Hazen & G. A. Edwards The fine structure of Histoplasma. Plate 5



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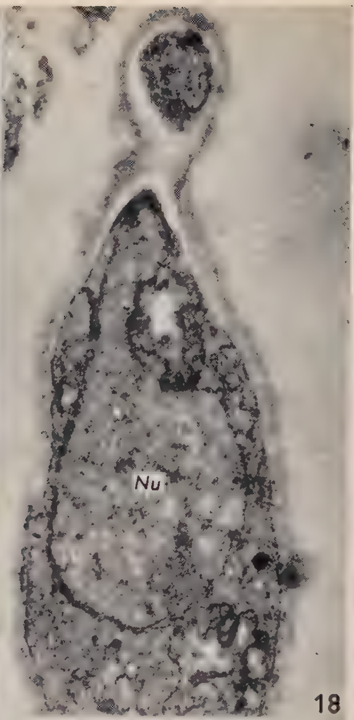

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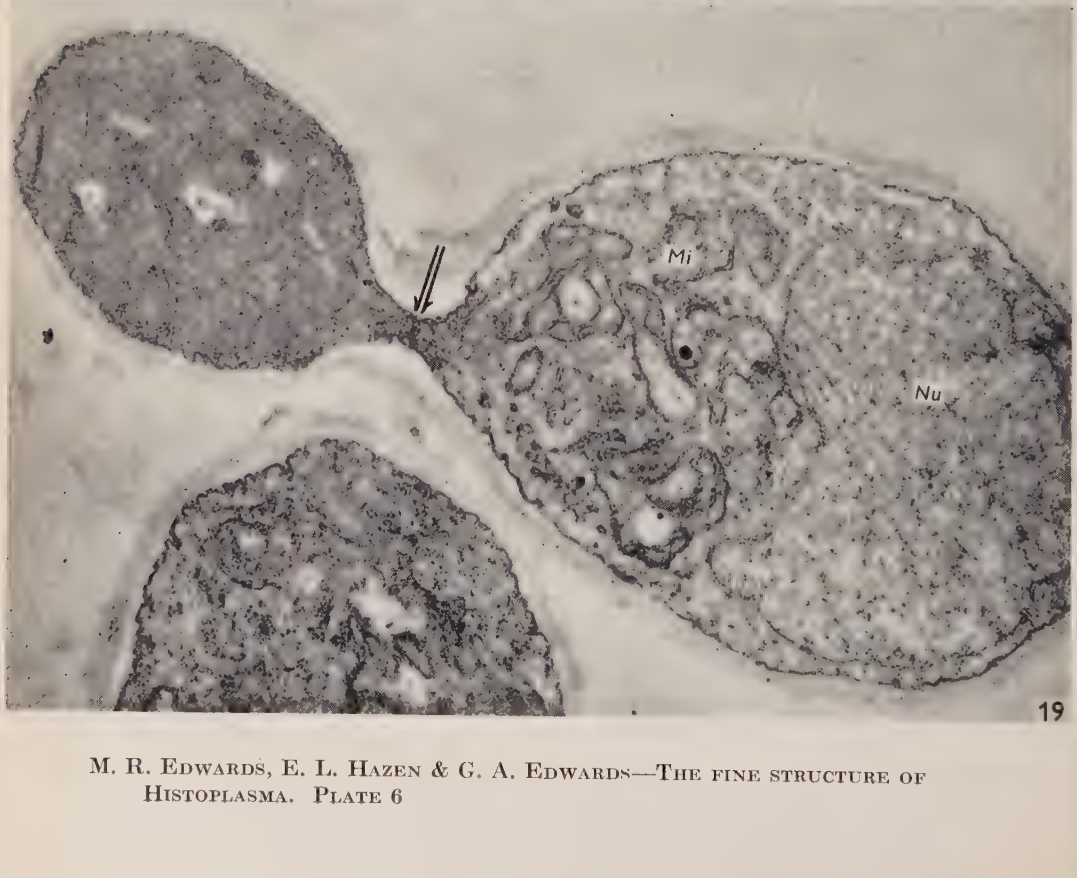

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M. R. Edwards, E. L. Hazen & G. A. Edwards—The fine structure of Histoplasma. Plate 6

PLATE 2

Fig. 3. *Histoplasma capsulatum.* The plasma membrane shows intense pinocytosis and appears connected to the endoplasmic reticulum. The external nuclear membrane is also connected with membranes of the endoplasmic reticulum. × 50,000.

Fig. 4. *Histoplasma capsulatum.* The outer membranes of mitochondria often appear continuous with the outer nuclear membrane or with the plasma membrane. × 45,000.

Fig. 5. *Histoplasma duboisii.* Dark cell whose nucleus is considerably less dense than is the cytoplasm. Two small mitochondria and several elements of the Golgi apparatus may be discerned in the cytoplasm. × 35,000.

Fig. 6. Light cell of *Histoplasma duboisii* with all organelles. The cell wall is thick and layered. The plasma membrane is relatively smooth. The mitochondria show few cristae. The endoplasmic reticulum comprises both sacs and vesicles. The nucleus possesses wide pores (arrow). × 35,000.

PLATE 3

Fig. 7. *Histoplasma duboisii.* A mitochondrion may possess long, complete, parallel cristae, usually in young cells. In upper portion of the cell there appear elements of the Golgi apparatus in cross section. × 45,000.

Fig. 8. *Histoplasma duboisii.* Young, dark cell with extreme pinocytotic activity of the plasma membrane. The cytoplasm contains a fat body, scattered granules and endomembranes, and two large mitochondria. Nuclear pores (arrows) are indicated. × 35,000.

Fig. 9. *Histoplasma duboisii.* Light cell showing mitochondrion with circular cristae. × 45,000.

Fig. 10. *Histoplasma capsulatum.* Early stage of the budding process. The cell wall is thickened around the bud and the plasma membrane in the bud area is extremely pinocytotic. × 45,000.

PLATE 4

Fig. 11. *Histoplasma capsulatum.* In early budding the nucleus is seen near the bud area. Mitochondria usually follow nucleus. × 32,000.

Fig. 12. *Histoplasma capsulatum.* As the bud increases in size, mitochondria appear in both mother cell and bud. × 32,000.

Fig. 13. *Histoplasma capsulatum.* The neck of the bud is constricted. The nucleus of the mother cell is located close to the neck of the bud and nuclear material seems to be present also in the bud. × 30,000.

Fig. 14. *Histoplasma capsulatum.* A definitive nucleus is present in the bud (at right) as well as in the mother cell. Mitochondria, lipid bodies, and endomembranes are present in both cells. In the neck region, the two cells are separated by their plasma membranes. In the common cell wall between plasma membranes, a dark line (the splitting plate) and partial furrowing (arrow) may be seen. × 30,000.

PLATE 5

Figs. 15, 16. *Histoplasma capsulatum.* Last steps of the budding process. In both figures the bud shows all the elements of an adult cell, but still remains connected to the mother cell by the common cell wall. In fig. 15, the splitting plate (arrow) along which the two cells will part, appears as two intimately apposed, thickened lines. These, on separation, will presumably be the independent, dense and thin, outer layers of the cell walls of the respective cells. Fig. 15, × 40,000; fig. 16, × 35,000.

PLATE 6

Fig. 17. *Histoplasma duboisii.* Early stage of the budding process. The cell wall around the bud is considerably thickened, the bud proper has a high electron density and its plasma membrane shows pinocytotic vesicle formation. × 30,000.

Fig. 18. *Histoplasma duboisii.* Intermediate stage of the budding process. A protrusion of the nucleus of the mother cell runs towards the bud. A small amount of nuclear material appears also in the bud itself. × 27,500.

Fig. 19. *Histoplasma duboisii.* The bud has a high electron density and its mitochondria are large with parallel cristae. Note the double plasma membrane formed in the splitting zone (double arrows) of the neck of the bud. × 35,000.

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Gene Controlled Resistance and Sensitivity to Caffeine and Nicotine in Saccharomyces

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SUMMARY: A significant extension in the variety of gene-markers available in Saccharomyces has been achieved by exploiting resistance and sensitivity to a variety of poisons. Although resistance to many poisons was obviously heritable, gene-control could not be demonstrated, presumably because other genes modified the phenotypes. Gene-control of resistance and sensitivity for both nicotine and caffeine was demonstrated by tetrad analysis and by the observation of regular Mendelian segregation of the characters among the progeny of closely related hybrids.

The project of constructing maps in Saccharomyces began with the exploitation of gene-markers controlling fermentation of various carbohydrates and was extended to include nutrilite requirements for various B-vitamins, amino acids and nucleic acid components. In this programme it was planned to use nutrilite requirements which could be diagnosed in any combination in the progeny of hybrids, but only a limited number of these are available, because substitution is relatively common. B-vitamin requirements have the disadvantage that the vitamin must be used in very low concentrations. About twenty suitable gene markers have been discovered in these categories and incorporated into the breeding stock. The search for new genetic markers has led to considering the possibility of using resistance and sensitivity to various poisons, since resistance and sensitivity to specific poisons seems to be a highly specific characteristic, and may make available a larger number of gene-markers than the nutritional or fermentative markers.

METHODS

Three procedures for diagnosing resistance and sensitivity were used.

(1) The test-tube method in which the organisms are inoculated into a tube containing a defined (or a complete) nutrient medium to which a specified amount of the toxic agent has been added (Middlekauff *et al.* 1957). In this procedure, a numerical value for the amount of growth can be obtained by readings made by optical density methods, but repeated tests do not always give the same results because of mutation and selection.

(2) The disk method in which paper disks (saturated with solutions containing different concentrations of the toxic agent) are placed on an agar surface freshly inoculated with the organisms to be tested is used with poisons that are not readily soluble or which precipitate or colour the medium. The tube method

is limited to a critical low concentration of poison which will permit growth to begin (because all the organisms in the tube are exposed equally to the toxic agent); but with the disk method it is possible to use disks saturated with concentrations of poison from 5 to 50 times stronger than could be used in a tube. The disk method enables one to observe the frequency of resistant secondary colonies in the clear zone (Pl. 1). In addition, agar overlay containing triphenyltetrazolium chloride (TTC) can be applied to the colonies to determine whether or not 'respiratory deficiencies' have been produced by the poison. Pink (adenine-requiring) cultures turn white or light brown when they become respiratory deficient, and in the disk method one may detect the occurrence of respiratory deficiency (due to the poison) without the TTC agar overlay. The disk method is used to discriminate between resistance and sensitivity to cadmium, propamidine, actidione and nicotine, and the tube method is used to discriminate between resistance and sensitivity to cadmium, actidione, caffeine and cobalt.

(3) The toxic agent may be incorporated into the agar and this method is used in distinguishing between resistance and sensitivity to copper. Copper is added to a full complex medium and the plate method permits the observation of secondary colonies (Ashida & Imai, 1956; Brenes-Pomales, Lindegren & Lindegren, 1955).

Diagnostic criteria of resistance to poisons

A few of the problems involved in diagnosing the genotypes of resistance and sensitivity must be considered. Cultures in the various pedigrees to be examined must be maintained on a complete complex (natural) nutrient medium which does not contain any known toxic agent. Cultures must not be recovered and placed in stock after the exposure to the poison, since exposure to toxic agents might lead to the overgrowth of the sensitive culture by resistant forms and to the development of double mutants in the resistant culture.

The diagnostic concentration must be one that kills all the sensitive organisms and permits all the resistant ones to survive and grow. Too high a concentration will kill both resistant and sensitive organisms and too low will permit resistant and sensitive organisms to grow and thus confuse the genetical analysis. It is interesting that more of the cultures survived and grew than failed to grow at an intermediate concentration, indicating that most organisms used were able to adapt to moderate concentrations of poisons.

Sibling cultures usually showed significant differences in sensitivity and resistance when grown in intermediate concentrations of poison, and replicate tests of resistance and sensitivity were usually reproducible, indicating that resistance and sensitivity were heritable traits.

With most poisons (propamidine, propamidine isethionate, mycostatin, salicylanilide, undecylenic acid, *p*-nitrophenol, 8-hydroxyquinoline, juglone, mercuric chloride and silver nitrate) regular monohybrid segregations were not discovered despite the fact that the characters were obviously inheritable, as inferred from the reproducibility of the tests for resistance and sensitivity. One would only expect regular monohybrid ratios if the modifiers of the genes of resistance and sensitivity produced only trivial effects on the phenotype and if the mutation rate were relatively low. It was surprising, therefore, to discover several instances of monohybrid segregation of resistance and sensitivity for several different toxic agents in unselected pedigrees.

Failures to replicate the diagnosis were fewer than might have been expected, and were usually interpreted to indicate that the selection of a resistant variant had occurred (Middlekauff *et al.* 1956).

Secondary colonies

Resistant cultures grew right up to the edge of the disk, but sensitive cultures left a clear zone around the disk. The clear zones around the disks of some sensitive cultures contained secondary resistant colonies. Sensitive cultures could be differentiated into those in which resistant variants occurred and those in which resistant variants did not occur. The frequency of secondary colonies depended on the toxic agent. Secondary colonies were common among copper-sensitive, propamidine-sensitive and cadmium-sensitive cultures, but this may be characteristic of the specific genes or genomes which have been studied. The first actidione-sensitive cultures showed large numbers of secondaries, but the more recent double-mutant controlling actidione-resistance produced fewer secondaries.

A sensitive culture which produces secondary colonies in the clear zone of a plate with the disk method would be expected to produce delayed secondary growth in the test tube and thus be incorrectly diagnosed as a resistant. This problem was analysed in detail in the study of resistance and sensitivity to cadmium (Middlekauff *et al.* 1956). Resistant cultures which grew rapidly and produced turbidity in 1 or 2 days were easy to distinguish from sensitive cultures which remained clear, i.e. failed to show growth after 10 or more days. Cultures in which growth occurred slowly after about 5 days presented a different problem. Such cultures were assumed to have been originally sensitive and to be distinguished from those which could not grow at all in the presence of the toxic agent, by the possession of an intermediate allele between dominant and the recessive; this was given the symbol of the sensitive allele with the superscript M. This designation is arbitrary since a sensitive culture with a very low mutation rate would be classified as exhibiting the recessive phenotype while one with a high mutation rate would be classified as exhibiting the dominant phenotype.

RESULTS

Inheritance of resistance to caffeine

Fries & Kihlman (1948), Demerec, Bertani & Flint (1951), and Novick & Szilard (1952) studied the effects of caffeine on the induction of hereditary, presumably gene-controlled, nutrilite requirements in fungi and bacteria and established the limits of caffeine toxicity. Fries & Kihlman discovered that 0·05 % (w/v) caffeine was slightly toxic to Ophiostoma and that all conidia were killed at a concentration of 0·2 %. Novick & Szilard grew the colon bacillus at a concentration of 0·015 % caffeine in the chemostat, presumably because this concentration did not inhibit its growth significantly.

The first discovery of saccharomyces cultures differing in resistance and sensitivity to caffeine was made during a survey of haploid cultures isolated from various pedigrees in previous breeding experiments. Many of the tetrads from these pedigrees showed 2:2 segregations of resistance to caffeine (using tubes of diagnostic medium containing 0·1 % caffeine). These cultures were used as the source of the pedigrees shown in Fig. 1. Unambiguous segregations were obtained when each haploid culture was tested in two different tubes, one containing 0·2 and one containing 0·4 % caffeine, as compared with the control containing no added caffeine. Many of these tests were confirmed by duplicate tests. Both the 0·2 and 0·4 % caffeine tubes gave comparable results. Mating of sensitive by sensitive, and resistant by resistant, produced progeny in which all offspring resembled the parent culture, thus confirming gene control of resistance to caffeine sensitivity (Fig. 1).

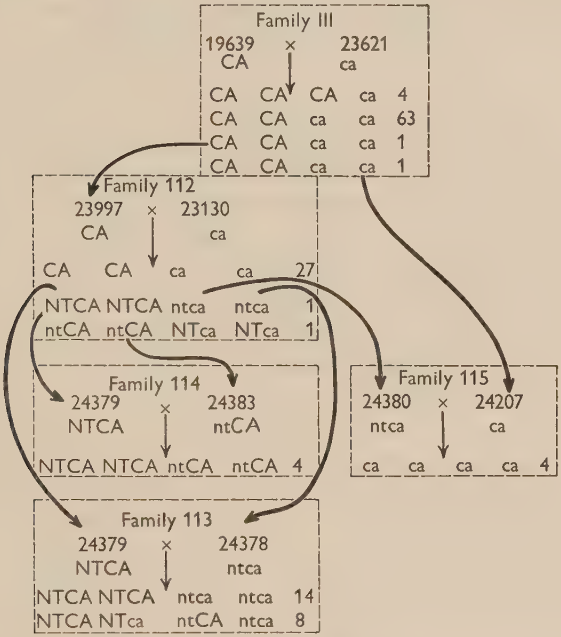

Family III
19639 × 23621
CA ca
CA CA CA ca 4
CA CA ca ca 63
CA CA ca ca 1
CA CA ca ca 1
Family 112
23997 × 23130
CA ca
CA CA ca ca 27
NTCA NTCA ntca ntca 1
ntCA ntCA NTca NTca 1
Family 114
24379 × 24383
NTCA ntCA
NTCA NTCA ntCA ntCA 4
Family 115
24380 × 24207
ntca ca
ca ca ca ca 4
Family 113
24379 × 24378
NTCA ntca
NTCA NTCA ntca ntca 14
NTCA NTca ntCA ntca 8

Fig. 1. A pedigree revealing regular Mendelian segregations in tetrads of saccharomyces hybrids heterozygous for genes controlling resistance to sensitivity to caffeine (CA, ca) and/or nicotine (NT, nt).

Gene control of resistance to nicotine

A series of cultures were sampled from Family 111 and indicated that this family was heterozygous for sensitivity and resistance to nicotine and one resistant and one sensitive member of this family were selected as parents for Family 114. The disk method was used in diagnosing resistance to nicotine because nicotine both darkens and precipitates the culture medium and cannot, therefore, be used in broth (Fig. 1). Disks were placed in solutions containing 1, 3, 5 and 10 % (w/v) nicotine in water. These disks were then placed on nutrient agar plates previously spread with one of the segregant cultures. Diagnosis of resistance and sensitivity was achieved within 24 hr. by inspection of the zone of inhibition. The use of four different concentrations of nicotine minimized ambiguity, since the series showed a progressive difference in size of the clear zone surrounding each disk. Such precipitation as occurred did not interfere with the diagnostic concentration established at the perimeter of diffusion.

The pedigrees

Family 111 produced sixty-five 2:2 tetrads and four 3:1 tetrads for resistance to caffeine. Family 112 was produced by mating a caffeine-resistant segregant from Family 111 by a caffeine-sensitive segregant from Family 108 and produced 29 tetrads, all of which yielded 2 caffeine-resistant and 2 caffeine-sensitive segregants. A caffeine-sensitive segregant from Family 112 was mated with another caffeine sensitive from Family 111, and produced 4 tetrads (Family 115), all containing only caffeine-sensitive progeny. Family 114 was produced by mating 2 caffeine-resistant clones derived from Family 112 and produced 4 tetrads all of which contained only caffeine-resistant segregants.

Sample cultures of Family 112 (which was heterozygous for genes controlling caffeine-sensitivity) revealed several tetrads containing 2 nicotine-resistant and 2 nicotine-sensitive segregants. One recombination tetrad of the genes controlling caffeine-resistance and nicotine-resistance was discovered. Family 114 was produced by mating a nicotine-resistant by nicotine-sensitive and produced 4 tetrads containing 2 nicotine-sensitive and 2 nicotine-resistant offspring. Family 113 was heterozygous for resistance to both caffeine and nicotine and produced 22 tetrads in which regular segregation for these two genes occurred in every tetrad. Fourteen tetrads carried the genes in the parental combination and 8 tetrads were tetratype.

These data established gene-control of resistance and sensitivity for both caffeine and nicotine. The analysis of tetrads which were heterozygous for other genes has served to locate the new genes on a chromosome carrying eight other markers. This chromosome will be described in detail subsequently (Lindegren *et al.* 1959).

The writers wish to acknowledge the technical assistance of Mr Bong Kuk Lee. This investigation was supported by a research grant from the Illinois Division of the American Cancer Society.

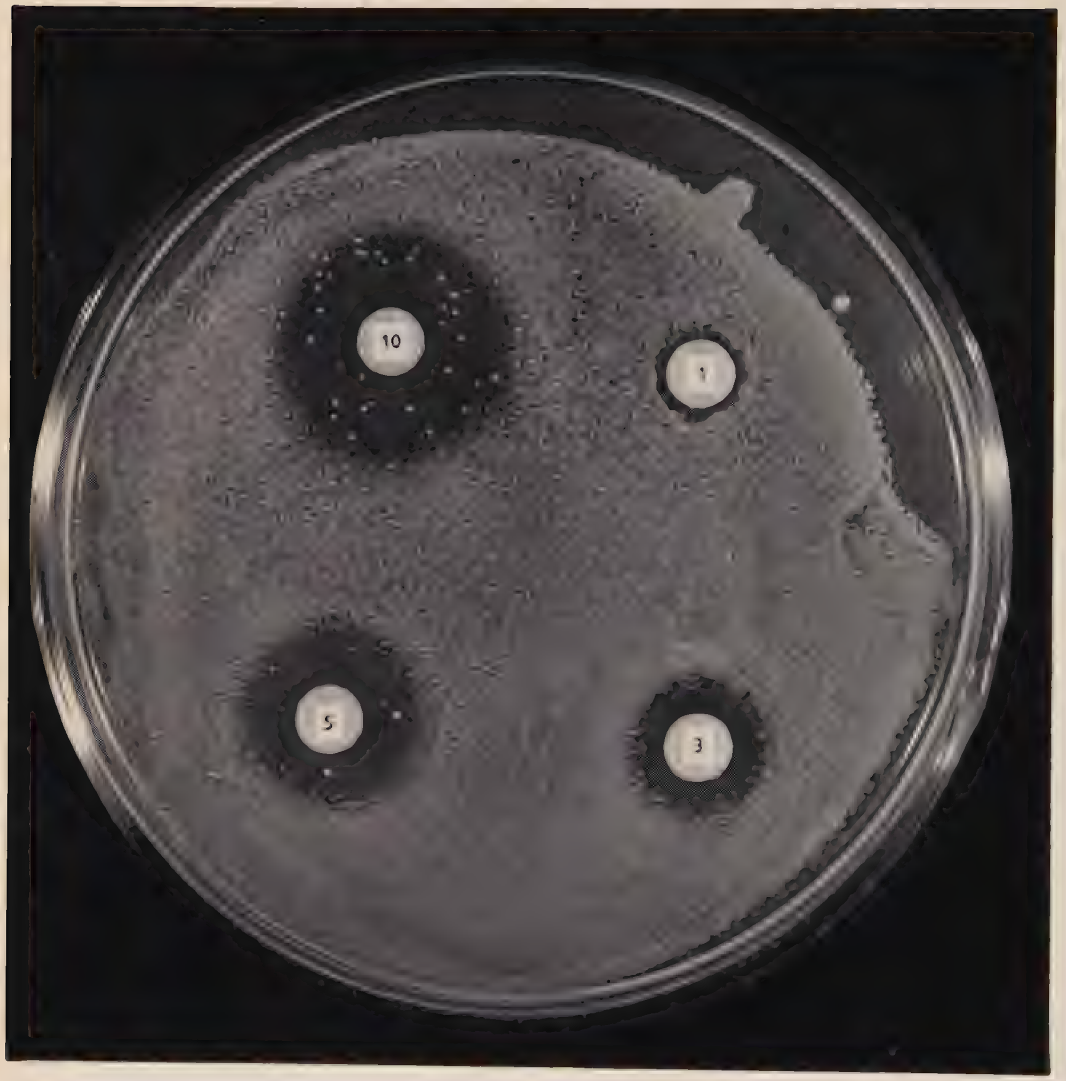
REFERENCES

Ashida, J. & Imai, M. (1956). Origin of copper resistant cell studies on the adaptation of yeast to copper. XV. *Bot. Mag. Tokyo*, **69**, 560.

Brenes-Pomales, A., Lindegren, G. & Lindegren, C. C. (1955). Gene control of copper sensitivity in Saccharomyces. *Nature, Lond.* **176**, 841.

Demerec, M., Bertani, G. & Flint, J. (1951). A survey of chemicals for mutagenic action on *E. coli*. *Amer. Nat.* **85**, 119.

Journal of General Microbiology, Vol. 20, *No.* 3



C. C. Lindegren, G. Lindegren & S. Desborough—New gene-markers in saccharomyces. Plate 1

(*Facing p.* 509)

FRIES, N. & KIHLMAN, G. (1948). Fungal mutations obtained with methyl xanthines. *Nature, Lond.* **162**, 573.

LINDEGREN, C. C., LINDEGREN, G., SHULT, E. & DESBOROUGH, S. (1959). A preliminary report on the chromosome maps of Saccharomyces (in the Press).

MIDDLEKAUFF, J. E., HINO, S., YANG, SING-PING, LINDEGREN, G. & LINDEGREN, C. C. (1956). Gene control of resistance *vs.* sensitivity to cadmium in Saccharomyces. *J. Bact.* **72**, 796.

MIDDLEKAUFF, J. E., HINO, S., YANG, SING-PING, LINDEGREN, G. & LINDEGREN, C. C. (1957). Gene control of resistance *vs.* sensitivity to actidione in Saccharomyces. *Genetics*, **42**, 66.

NOVICK, A. & SZILARD, L. (1952). Anti-mutagens. *Nature, Lond.* **170**, 926.

EXPLANATION OF PLATE

The effect of placing paper disks saturated with solutions of nicotine on the growth of nicotine-sensitive saccharomyces previously sown on an agar plate. The strength of the different nicotine solutions is indicated on the paper disk. The resistant secondary colonies appearing in the clear zone do not complicate the analysis as they might if the test were performed in a broth tube.

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HAYWARD, A. C., FORSYTH, W. G. C. & ROBERTS J. B. (1959). *J. gen. Microbiol.* 20, 510–518

Synthesis and Breakdown of Poly-β-Hydroxybutyric Acid by Bacteria

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SUMMARY: The synthesis and breakdown of poly-β-hydroxybutyric acid in growing cultures of selected strains of Rhizobium, Spirillum and Pseudomonas are described. The occurrence of the polymer in root nodules of tropical and temperate leguminous plants is reported.

The occurrence of poly-β-hydroxybutyric acid in the genus *Bacillus* was reported by Lemoigne, Delaporte & Croson (1944), who were able to correlate the presence of prominent refractile granules in the cells with the isolation of a wax-like, chloroform-soluble, ether-insoluble polymer from the large-celled bacilli, *Bacillus megaterium*, *B. cereus*, *B. mycoides* and *B. anthracis*. Williamson & Wilkinson (1958) extracted the inclusions with an alkaline solution of sodium hypochlorite and proved that poly-β-hydroxybutyric acid was their major constituent. Lemoigne & Girard (1943) demonstrated the same polymer in *Azotobacter chroococcum*. Recently Forsyth, Hayward & Roberts (1958) recorded the presence of poly-β-hydroxybutyric acid in *A. vinelandii* and *A. agilis* and also in Rhizobium, certain pseudomonads and Chromobacterium, groups in which the presence of the polymer had not previously been suspected.

A considerable amount of work has been carried out on the chemical composition of the root nodules of leguminous plants and of bacteria isolated from them, yet there has been no positive chemical identification of the wax-like sudanophilic material known to occur in Rhizobium (Lewis, 1938). Moller (1892, cited by Lewis, 1938) studied the effect of various solvents on the refractile granular inclusions of Rhizobium and showed that they were insoluble in ether, but soluble in chloroform. He concluded that the waxy substance corresponded most nearly to cholesterol, which was also the conclusion of Muller & Stapp (1925, cited by Lewis, 1938) from a chemical analysis of bacteroids. Hopkins, Peterson & Fred (1929) extracted dried *Rhizobium meliloti* 100 with chloroform and ether, and showed that different samples of this organism contained between 10·2 and 21·7 % of the dry weight as a chloroform-soluble fat-like substance, which was isolated as a brittle mass having a melting point of 172–173° (Hopkins & Peterson, 1930).

In Spirillum several types of granule have been recognized. Volutin and 'fat' were described by Lewis (1940), though Williams & Rittenberg (1957) were unable to confirm that the 'fat' granules were sudanophilic. King & Beams (1942) subjected *Spirillum volutans* to ultracentrifugation and succeeded in displacing all the visible granules into the centrifugal region. They thus concluded that the granules, of which there were at least two types in the cell, were heavier than the protoplasm surrounding them. As only a minority of the granules refractory to staining with iron hematoxylin reduced osmic acid they concluded that these granules were not fat. With *Bacillus megaterium*, Williamson & Wilkinson (1958) provided convincing evidence that volutin and the inclusions containing poly-β-hydroxybutyric acid can be differentiated by staining techniques and have a separate identity. The evidence in the case of Spirillum is less conclusive.

The present study, and that reported earlier (Forsyth *et al.* 1958), were undertaken because it was suspected that poly-β-hydroxybutyric acid is more widely distributed in bacteria than previously realized, and may occur in the bacterial cell in massive concentrations when the organisms are grown under appropriate conditions. The effect of substrate concentration on polymer yield has been investigated.

METHODS

Organisms. The majority of bacteria used in this study were obtained from Miss M. B. Morris, Curator, Hankey Culture Collection at this Institute; the cultures of *Pseudomonas solanacearum* were freshly isolated from banana plants with 'Moko' disease and from wilted tomato plants. Cultures of *Rhizobium* spp. from the temperate zone were obtained through the courtesy of Dr G. Bond, Botany Department, University of Glasgow. Cultures of Spirillum were obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex, as follows: *Spirillum serpens*, NCIB 8658, and three large-celled, unidentified, spiral bacteria numbered I, II and III, isolated by micromanipulation of single cells from Hampton sewage. Several spiral bacteria were isolated by us from calcium lactate enrichment cultures as described by Williams & Rittenberg (1957).

An organism (strain B175) which produces a phenazine pigment, was also used and was typical of many similar strains isolated from Trinidad soils.

Methods of cultivation. A New Brunswick type rotary shaking machine was used at either 200 or 300 rev./min. The organisms were grown in 250 ml. conical flasks containing either 50 or 100 ml. medium. The temperature of the room housing the shaker varied between 26° and 29°.

For cultivation of the pseudomonads use was made of the salts solutions (i) and (ii) described by Powell (1958). Concentrated glucose solutions in distilled water were sterilized separately by autoclaving, and solutions of sodium hydroxybutyrate (British Drug Houses Ltd., lab. reagent) by Seitz filtration; they were then added with sterile precautions to the basal salts medium.

Cultures of Rhizobium were grown in a modification of Allison's medium prepared as described by Wilson & Knight (1952). D-Mannitol (20 g./l.), DL-asparagine (1 g./l.) and Difco Yeast Extract (1 g./l.) were added to the solution of salts, together with 1 ml./l. of a 0·9 % (w/v) solution of ferric chloride. Most but not all rhizobia grew well in this medium.

Spirillum serpens 8658 was grown in Allison's medium as described above, but with 2 % (w/v) lactic acid instead of D-mannitol.

Staining methods. Lipid inclusions in smears and crushed root nodules were stained with an ethanolic solution of Sudan Black B (G.T. Gurr Ltd., London) according to the method of Burdon (1946).

Dry-weight determinations and polymer extraction. Whole cultures were centrifuged and usually washed once in distilled water before drying overnight at 105°. Dried deposits were then scraped out into a weighing bottle, weighed, dried again for several hours, weighed again, pulverized, extracted twice or three times with chloroform and the polymer precipitated by dilution with ether (Forsyth *et al.* 1958). Poly-β-hydroxybutyric acid precipitates were dried at 105° overnight before weighing.

Organic matter and keto acids. Keto acids were determined in the supernatant liquors by the method of Cavallini & Frontali (1954), and organic matter by the method of Johnson (1949). Melting points were taken on a Kofler block, of the temperature at which melting began. Melting points were uncorrected.

RESULTS

Rhizobium

Washed and dried root nodules were pulverized and extracted with chloroform whenever they were available in sufficient quantity for a quantitative examination; otherwise nodules were crushed on to a slide and stained with Sudan Black B. Results are summarized in Table 1. Most isolates of Rhizobium produced a copious polysaccharide capsule on agar or in shake-flask cultures, which invariably made centrifugation difficult. One strain, B142, from Pueraria was chosen for further study because it produced little or no capsular gum. Duplicate shake flask cultures in Allison's medium were removed at 2-day intervals and the dried organisms from each separately extracted with chloroform. The results (Fig. 1) indicate that massive quantities of poly-β-hydroxybutyric acid were formed during the growth of strain B142. Many other rhizobia produced

Table 1. *Occurrence of poly-β-hydroxybutyric acid in root nodules of leguminous plants*

Source of root nodules	Wt. of dried nodules (mg.)	Wt. of isolated polymer (mg.)	% dry wt.	M.P. (° C.)
Immortel (*Erythrina poeppigiana*)	*(i) 2830	229	8·1	170
	(ii) 2733	143	5·2	N.M.
String bean (*Vigna unguiculata sesquipedalis*)	1896	137·5	7·3	172
Canavalia maritima	2022	239	11·8	167
Mimosa pudica	*(i) 900	54	6·0	N.M.
	(ii) 332	Trace	<1·0	N.M.

Flocculent, chloroform soluble, ether insoluble, material was also obtained from small samples of the root nodules of lupin (*Lupinus*), kudzu (*Puereria*) and gorse (*Ulex*). Crushed nodules of bush vetch (*Vicia sepum*) and *Crotalaria*, which were not sufficient for extraction, were strongly sudanophilic.

* (i) and (ii) are samples from different localities. N.M. = not measured.

comparable yields. Cultures of B 142 also grew vigorously and with production of polymer when the D-mannitol of the basal medium was replaced by glucose, glycerol or sodium hydroxybutyrate.

The following strains of rhizobia obtained from the Botany Department, University of Glasgow, were shown to contain poly-β-hydroxybutyric acid: Field bean (strain S), Lucerne (strain AH), *Trifolium* (strains 21, F, 49 R, 101 and 20272), *Phaseolus* (strain 5 B) and *Pisum* (strain 317). All were grown in Allison's medium on the rotary shaker for periods of 3–14 days.

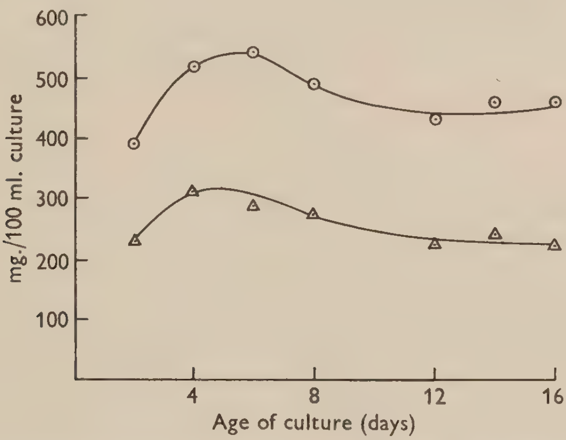

Fig. 1. Poly-β-hydroxybutyric acid content and dry weight of Rhizobium strain B 142 grown in a modification of Allison's medium. ⊙—⊙, Dry weight of organisms; △—△, weight of poly-β-hydroxybutyric acid. 100 ml. medium/250 ml. conical flasks on a rotary shaker at 200 rev./min. Initial concentration of D-mannitol, 20 g./l.

Two cultures of *Rhizobium japonicum* from soyabean (*Glycine*) were obtained through the courtesy of Dr P. S. Nutman, Soil Microbiology Department, Rothamsted Experimental Station. Both strain 507, which was ineffective for the fixation of nitrogen, and strain 505 (effective) produced poly-β-hydroxybutyric acid during growth in a medium containing L(+) arabinose as major source of carbon.

Spirillum

The observations of Lewis (1940) led us to suspect that the genus *Spirillum* Ehrenberg would include organisms capable of synthesizing large quantities of poly-β-hydroxybutyric acid. The medium used initially contained the following materials (g./l. of distilled water): Oxoid peptone, 5·0; Difco yeast extract, 5·0; calcium lactate, 20. The pH value was adjusted to *c.* 7·6 with 40 % (w/v) NaOH. Heavily inoculated conical flasks of medium were then grown on a reciprocating shaker. After incubation for 16–22 hr. the polymer was extracted. The cultures of *Spirillum serpens* and strains I, II and III from Hampton sewage were all found to contain between 10 and 20 % poly-β-hydroxybutyric acid. After incubation for 40–60 hr. each culture contained very much less of the polymer; in two cultures none was detectable after 60 hr. of incubation. Evidently the polymer was formed in young cultures, in which the spiral bacteria contained a chain of refractile granules, and rapidly disappeared from old cultures which appeared uniform in smears stained with dilute carbol fuchsin. It became apparent that the calculated % yields of polymer obtained in these preliminary experiments with calcium lactate were too low because a heavy inorganic precipitate, presumably calcium carbonate, was present at an early stage of growth.

Spirillum serpens NCIB 8658 grew well in Allison's medium containing lactic acid as major carbon source. The results of a typical experiment are shown in Fig. 2. The concentration of poly-β-hydroxybutyric acid rose to a maximum of 34·2 % of the dry weight at 38 hr. and fell to 7·5 % at 110 hr. Similar results were obtained when glycerol or sodium hydroxybutyrate replaced the lactate in the basal medium.

Pseudomonas

Pseudomonas antimycetica (Thaysen & Thaysen, 1953) grew heavily in the salts medium supplemented with 2 % lactic acid and produced a maximum of 51·5 % polymer in the dry cell weight.

The rough colony form of *Pseudomonas solanacearum* grew slowly with synthesis of polymer in the salts medium supplemented with either glycerol or β-hydroxybutyrate. The appearance of the cells of the smooth colony form of *P. solanacearum* in 'fat' stained smears was very characteristic, the greater part of the cell staining intensively with Sudan Black B. According to Dowson (1949) this plant pathogen does not stain readily by the usual bacteriological methods. In smears stained with dilute carbol fuchsin there was a bipolar effect, which has not previously been associated with the presence of 'fat'.

Non-pigmented Gram-negative rods having an oxidative metabolism of carbohydrates have been isolated in a lactate + salts enrichment medium, and a few of these have proved to contain poly-β-hydroxybutyric acid. One type isolated twice from river water resembled *Vibrio*; the others are probably pseudomonads. Green pigmented pseudomonads have so far proved to be devoid of the polymer (Forsyth *et al.* 1958) but experiments with selective media are continuing.

Strain B175

This organism, typical of many similar strains isolated from Trinidad soils, has polar flagella and produces a phenazine pigment (Morris & Roberts, unpublished). Its classification is not yet settled but it is probably a pseudomonad.

Strain B175 grew heavily and produced poly-β-hydroxybutyrate in the salts medium supplemented with glucose, glycerol, lactate, citrate, succinate or β-hydroxybutyrate as sole source of carbon. The extent of poly-β-hydroxybutyrate accumulation was conditioned by the initial concentration of glucose (Table 2); in 2 % β-hydroxybutyrate + salts medium the concentration of polymer fell from a maximum of 51·3 % at 62·5 hr. to 3·7 % at 158·5 hr. (Fig. 3). The results in Fig. 3 and Table 3 are typical of several experiments in which strain B175 was grown in 2 % or 0·5 % lactic acid or hydroxybutyrate media. In 2 % lactic acid medium α-ketoglutarate accumulated but not at the stage of culture when the polymer was rapidly being degraded. There was no accumulation of keto acids at any stage of growth at the lower concentration of lactic acid. Organic matter decreased smoothly. Our results thus support the conclusions of Macrae & Wilkinson (1958) that under aerobic conditions carbon dioxide and water are the main products of breakdown of the polymer.

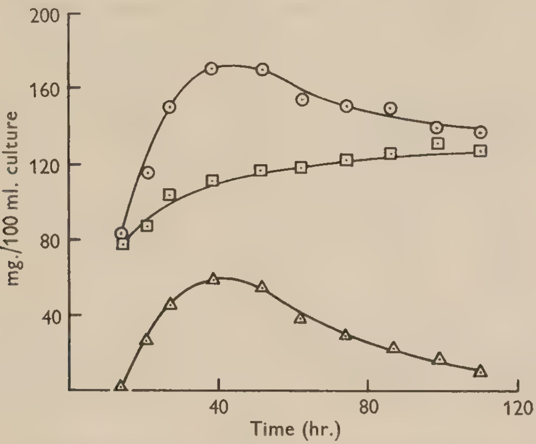

Fig. 2. Poly-β-hydroxybutyric acid content and dry weight of cells of *Spirillum serpens* NCIB 8658 grown in a modification of Allison's medium. ⊙—⊙, Dry weight of organism; △—△, weight of poly-β-hydroxybutyric acid; ⊡—⊡, weight of cells—weight of polymer. Inoculum: 1 ml./flask of a 5-day shake-flask culture. Duplicate flasks removed periodically from rotary shaker (200 rev./min.). Initial concentration of sodium lactate equiv. to 20 mg. lactic acid/ml.; 100 ml. medium/250 ml. shake flask.

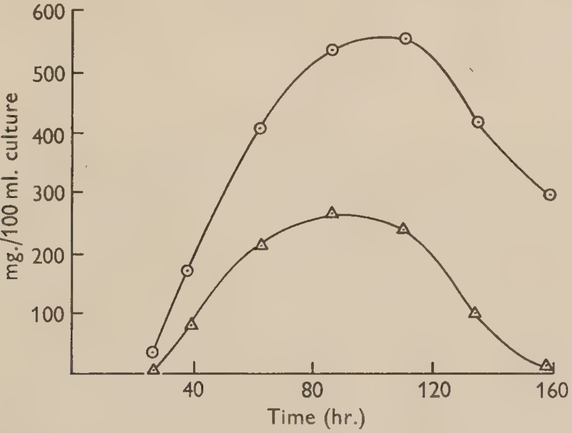

Fig. 3. Poly-β-hydroxybutyric acid content and dry weight of organism B175 grown on a sodium hydroxybutyrate + salts medium. ⊙—⊙, dry weight; △—△, weight of poly-β-hydroxybutyric acid. 100 ml. medium/250 ml. conical flasks on a rotary shaker (200 rev./min.) Initial concentration of sodium hydroxybutyrate, 20 mg./ml.

There was no evidence in either growing cultures or resting cell experiments that organism B175 could break down finely divided poly-β-hydroxybutyric acid when this was added extracellularly.

Table 2. *The effect of initial concentration of glucose on the poly-β-hydroxybutyric acid content of organism* B175

Five ml. of 20% or 5% (w/v) sterile glucose solutions were added to 45 ml. of basal salts medium. Flasks were incubated on a rotary shaker at 300 rev./min.

	Medium contained					
	0·5% glucose	2% glucose	0·5% glucose	2% glucose	0·5% glucose	2% glucose
Age of culture (hr.)	mg. cell dry wt./100 ml. culture		mg. polymer/100 ml. culture		% polymer/cell dry weight	
23	203	108	5·0	Trace	2·5	<1
46	172	509	Trace	162	<1	31·7
92	126	531	0	85	0	16·0

Table 3. pH *value, keto acid, and residual organic matter content of supernatant liquors of cultures of organism* B175 *grown in a sodium hydroxybutyrate + salts medium*

Time (hr.)	pH value	Organic matter (%)	Ketoglutarate (μg./ml.)
0	*c.* 7·0	100	0
27·0	7·2	90	0
38·5	7·7	83	0
62·5	8·4	80	2
86·5	8·3	43	7·5
110·5	8·3	25	Trace
134·5	8·4	20	0
158·5	8·3	10	0

DISCUSSION

There are many descriptions in the literature of sudanophilic inclusions which resist staining with aniline dyes and which may well be deposits of poly-β-hydroxybutyric acid. Lewis, for example, wrote a series of papers on the cytology and life cycles of *Bacillus mycoides* (1934), Azotobacter (1937), Rhizobium (1938) and Spirillum (1940), and in each case described inclusions with these characteristic properties. Information is required on the identity of certain opaque structures seen in ultra-thin sections of bacteria under the electron-microscope, which it seems probable may be identical with deposits of poly-β-hydroxybutyric acid. Hashimoto & Naylor (1958) described such inclusions in *B. cereus* and Carr (1958) in *Azotobacter agilis*. The electronmicrographs of thin sections of *Rhodospirillum rubrum* prepared by Vatter & Wolfe (1958) show large spherical vacuoles which they described as corresponding to areas in whole cells which stained with Sudan Black B. Duchow & Douglas (1949) described refractile globules in the photosynthetic organism *Rhodomicrobium vannielii* which stained with Sudan Black B and were assumed to be fat. Grula & Hartsell (1954) studied the intracellular structure of the stalked bacterium *Caulobacter vibrioides*; intensely sudanophilic 'fat' vacuoles were described. In view of the similarity of these descriptions to those of structures in bacteria which are now known to contain poly-β-hydroxybutyric acid, reinvestigation of the possibility that they do contain this substance is required.

Umezu (1940) described the occurrence of a chloroform-soluble white amorphous substance, melting at 169°–172°, which constituted 0·54 % of the dried glanders organism, *Malleomyces mallei.* Worley & Young (1945) subjected several strains of this pathogen to fat-staining techniques; Sudan Black B was particularly effective for the staining of 'lipid' granules which failed to stain readily with the usual aniline dyes. It seems probable that this strictly parasitic organism contains deposits of poly-β-hydroxybutyric acid.

Although solid poly-β-hydroxybutyric acid isolated from bacteria does not stain with Sudan Black B (Williamson & Wilkinson, 1958), we have noted that when dissolved in chloroform and applied to defatted filter-paper the polymer deposited in the paper readily takes up the fat stain. Under the same conditions we have found no reduction of the polymer by a 1 % solution of osmium tetroxide which apparently only oxidizes unsaturated fats (McClung, 1951, cited by Williams & Rittenburg, 1957).

Macrae & Wilkinson (1958) gave reasons for considering that in *Bacillus megaterium*, poly-β-hydroxybutyric acid is a storage compound. The fact that the polymer occurs in root nodules, as well as the synthesis and dissimilation of the material in growing cultures of Rhizobium, Spirillum and Pseudomonas, is in accordance with this suggestion. There have been reports that glycogen also occurs in root nodules as a storage compound (Bergerson, 1955).

The irregular occurrence of poly-β-hydroxybutyric acid in Pseudomonas suggests that the presence of this substance may be a valuable criterion in the classification of members of the genus, particularly the numerous plant pathogens. A wider study of Pseudomonas is to be undertaken in this laboratory to examine this possibility.

We are grateful to Dr V. C. Quesnel for provision of root nodules and identification of leguminous plants and Mr J. Spence, Department of Agriculture, Trinidad, for assistance in the identification of *Pseudomonas solanacearum.*

REFERENCES

Bergerson, F. J. (1955). The cytology of bacteroids from root nodules of subterranean clover (*Trifolium subterraneum* L.). *J. gen. Microbiol.* **13**, 411.

Burdon, K. L. (1946). Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *J. Bact.* **52**, 665.

Carr, L. B. (1958). Observations on the capsule of *Azotobacter agilis*. *J. Bact.* **76**, 119.

Cavallini, D. & Frontali, N. (1954). Quantitative determinations of keto-acid by paper partition chromatography. *Biochim. biophys. Acta*, **13**, 439.

Dowson, W. J. (1949). *Manual of Bacterial Plant Diseases.* London: Adam and Charles Black.

Duchow, E. & Douglas, H. C. (1949). *Rhodomicrobium vannielii* a new photoheterotrophic bacterium. *J. Bact.* **58**, 409.

Forsyth, W. G. C., Hayward, A. C. & Roberts, J. B. (1958). The occurrence of poly-β-hydroxybutyric acid in aerobic Gram-negative bacteria. *Nature, Lond.* **182**, 800.

GRULA, E. A. & HARTSELL, S. E. (1954). Intracellular structures in *Caulobacter vibrioides*. *J. Bact.* **68**, 498.

HASHIMOTO, T. & NAYLOR, H. B. (1958). Studies of the fine structure of microorganisms. I. A study of factors influencing the 'explosion phenomenon' in ultrathin sections of bacteria. *J. Bact.* **75**, 640.

HOPKINS, E. W. & PETERSON, W. H. (1930). The nature of the fat-like constituents of the alfalfa root nodule bacteria. *J. Bact.* **19**, 9.

HOPKINS, E. W., PETERSON, W. H. & FRED, E. B. (1929). The composition of the cells of certain bacteria with special reference to their carbon and their nitrogen content. *J. biol. Chem.* **85**, 21.

JOHNSON, M. J. (1949). A rapid micromethod for estimation of non-volatile organic matter. *J. biol. Chem.* **181**, 707.

KING, R. L. & BEAMS, H. W. (1942). Ultracentrifugation and cytology of *Spirillum volutans*. *J. Bact.* **44**, 597.

LEMOIGNE, M., DELAPORTE, B. & CROSON, M. (1944). Contribution à l'étude botanique et biochimique des bactéries du genre Bacillus. 2. Valeur du test des lipides β-hydroxybutyriques pour la characterisation des espèces. *Ann. Inst. Pasteur*, **70**, 224.

LEMOIGNE, M. & GIRARD, H. (1943). Réserves lipidiques β-hydroxybutyriques chez *Azotobacter chroococcum*. *C.R. Acad. Sci., Paris*, **217**, 557.

LEWIS, I. M. (1934). Cell inclusions and endospore formation in *Bacillus mycoides*. *J. Bact.* **28**, 133.

LEWIS, I. M. (1937). Cell inclusions and the life cycle of *Azotobacter*. *J. Bact.* **34**, 191.

LEWIS, I. M. (1938). Cell inclusions and the life cycle of *Rhizobia*. *J. Bact.* **35**, 573.

LEWIS, I. M. (1940). The genus *Spirillum* Ehbg. with special reference to cell inclusions and the chromidial theory. *J. Bact.* **40**, 271.

MCLUNG, C. E. (1951). *Handbook of Microscopical Technique*, 3rd ed. New York: Paul B. Hoeber, Inc.

MACRAE, R. M. & WILKINSON, J. F. (1958). Poly-β-hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *B. megaterium*. *J. gen. Microbiol.* **19**, 210.

MOLLER, H. (1892). Bemerkungen zu Frank's Mittheilung ueber den Dimorphismus der Wurzelknöllchen der Erbse. *Ber. dtsch. bot. Ges.* **10**, 242.

MÜLLER, A. & STAPP, C. (1925). Beiträge zur Biologie der Leguminosenknöllchen bakterien mit besonderer Berücksichtigung ihrer Artverschiedenheit. *Arb. biol. Abt. (Anst.—Reichsanst.), Berl.* **14**, 455.

POWELL, E. O. (1958). An outline of the pattern of bacterial generation times. *J. gen. Microbiol.* **18**, 382.

THAYSEN, A. C. & THAYSEN, I. (1953). Comirin, a new fungistatic antibiotic. *Rep. Proc. VI int. Congr. Microbiol., Rome*, **1**, 321.

UMEZU, M. (1940). *Rep. Inst. Sci. Res. Manchoukuo*, **4**, 273. (*Chem. Abstr.* **1941**, **35**, 1825.)

VATTER, A. E. & WOLFE, R. S. (1958). The structure of photosynthetic bacteria. *J. Bact.* **75**, 480.

WILLIAMS, M. A. & RITTENBERG, S. C. (1957). A taxonomic study of the genus *Spirillum* Ehrenberg. *Int. Bull. bact. Nomen. Taxon.* **7**, 49.

WILLIAMSON, D. H. & WILKINSON, J. F. (1958). The isolation and estimation of the poly-β-hydroxybutyrate inclusions of *Bacillus* species. *J. gen. Microbiol.* **19**, 198.

WILSON, P. W. & KNIGHT, S. G. (1952). *Experiments in Bacterial Physiology*, 3rd ed., p. 52. Minneapolis: Burgess Publishing Co.

WORLEY, G. & YOUNG, G. (1945). The glanders organism with reference to its cell inclusions. *J. Bact.* **49**, 97.

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Localization of Enzymes in *Bacillus megaterium*, Strain M

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SUMMARY: Protoplasts were prepared from cells of *Bacillus megaterium*, strain M, by lysozyme treatment in the presence of sucrose. The protoplasts were shocked osmotically and the lysate thus obtained was fractionated into membranous 'ghosts' and soluble protoplasm by means of differential centrifugations. The distribution of various enzymes between these two fractions was studied. The bulk of the cytochromes, the succinic dehydrogenase and the diphosphopyridine nucleotide oxidase of the lysate was recovered in the 'ghost' fraction. On the other hand, the soluble protoplasm contained most of the isocitric dehydrogenase, the catalase, the hexokinase and the acid phophatase of the lysed bacteria. Considerable amounts of malic dehydrogenase, lactic dehydrogenase and fumarase were found in both the 'ghosts' and the soluble protoplasm. None of the enzymes studied was localized in the ribonucleic acid-containing particles of the bacterial protoplasm.

Many of the enzymes of animal cells are localized in subcellular structures such as mitochondria and microsomes. It is doubtful whether a similar state of affairs prevails in bacterial cells. Numerous investigations (reviewed by Alexander, 1956) have made it clear that, in extracts obtained by a mechanical or sonic disintegration of bacterial cells, several enzymes are associated with the sedimentable particles. Without additional evidence it cannot be settled whether these particles exist as such in the intact cells or whether they represent the remnants of larger, subcellular structures. In some cases at least, however, the 'differential release technique' devised by Marr & Cota-Robles (1957) may be useful for discriminating between these two possibilities.

Some of the hazards of the mechanical and sonic disintegration procedures are avoided when the bacterial cells are degraded enzymically or autolytically. Thus bacterial 'ghosts' (probably mainly cytoplasmic membranes) have been isolated and characterized after treating *Bacillus megaterium*, strains KM and M, with lysozyme (Weibull, 1953*a*, *b*; Vennes & Gerhardt, 1956; Weibull, 1956; Storck & Wachsman, 1957; Weibull & Bergström, 1958). 'Ghosts' of *Micrococcus lysodeikticus* have been studied in the same way (Gilby, Few & McQuillen, 1958). Mitchell & Moyle (1956*a*, *b*) used an autolytic procedure for the isolation of a plasma-membrane fraction from *Staphylococcus aureus*. Spiegelman, Aronson & Fitz-James (1958) reported the isolation of bacterial nuclei by means of the enzymic degradation of the cytoplasmic membrane.

The present study deals with the distribution of certain enzymes between the 'ghosts' and the rest of the protoplasm of *Bacillus megaterium*, strain M. This study thus links up closely with the investigation of Storck & Wachsman (1957). These workers studied the KM strain of the same organism. However, it was considered of some interest to compare the enzymic properties of two strains of the same species. Since, moreover, the chemical properties of the 'ghosts' of the M strain have been studied in some detail (Weibull, 1957; Weibull & Bergström, 1958), it has been possible to bring into relation the enzymic and chemical properties of one and the same subcellular structure.

METHODS

Organism, growth conditions and harvesting. Bacillus megaterium, strain M (Baumann-Grace & Tomcsik, 1957) was grown in the medium described by Gladstone & Fildes (1940). Fernbach flasks having a volume of 2–8 l. and containing 250–1000 ml. medium were inoculated with the organism and incubated for 16 hr. at 30° on a rotary shaker (speed of shaker 100 rev./min.). The bacteria, then being in the stationary growth phase, were harvested by centrifugation and suspended in 0·02 M-phosphate buffer (pH 7). The concentration of the bacteria in the suspension was made about 4 times that in the growth medium at harvesting.

Preparation of bacterial fractions. To each 225 ml. of bacterial suspension, prepared as described above and brought to room temperature, 75 ml. 2 M-sucrose and 6 ml. 1 % (w/v) lysozyme were added. Protoplast formation was completed within about 30 min. The protoplasts were centrifuged for 20 min. at 20,000 ***g*** (Spinco model L preparative ultracentrifuge with the no. 30 rotor) and were then resuspended in 100 ml. of cold (4°) distilled water containing 0·2 mg. crystalline deoxyribonuclease. Because of the released deoxyribonucleic acid, the lysate thus obtained was at the beginning rather viscous, but after *c.* 15 min. showed the viscosity of a normal aqueous solution. A sample of the lysate (L) was withdrawn and the remainder was subjected to the fractionation procedure outlined in Fig. 1. During this fractionation the temperature of the bacterial preparations was kept below 5°.

Thus three kinds of bacterial preparations were obtained: total lysate (L), 'ghosts' (G) and soluble protoplasm (S). The volumes of these preparations were adjusted so that each of them contained (per ml.) material from the same amount (*c.* 15 mg.) of bacteria. A reconstituted lysate (S + G) was obtained by mixing equal amounts of S and G. Compared with the original lysate, this reconstituted system thus contained the equivalent of half the amount of bacterial dry matter/ml. This was taken into account when calculating the figures in Tables 3 and 4. The bacterial preparations were used immediately for enzymic assays.

Enzyme assays. All assays except the hexokinase and phosphatase determinations were carried out at room temperature (20–22°). The enzymic activities of the various bacterial fractions were determined in the shortest possible time, each enzyme being assayed in turn. Prior to the determinations, the enzyme solutions were kept in an ice bath. Samples (1 ml.) were allowed to warm up for 1 min. at room temperature before being used in the tests.

The method for assaying succinic, malic, lactic, isocitric and formic dehydrogenases was essentially that used by Storck & Wachsman (1957). Dichlorophenol indophenol was, however, replaced by potassium ferricyanide as the hydrogen acceptor. The reaction mixture contained: 0·3 ml. 0·5 M-phosphate; 0·1 ml. 0·15 M-$MgSO_4$; 0·2 ml. 0·15 M-KCN; 0·2 ml. 0·008 M-$K_3Fe(CN)_6$; 1 ml. substrate; 1 ml. enzyme solution; water to 3·0 ml. The final pH value of the mixture was 7·0. The following solutions were used as substrates: 0·3 M-sodium DL-lactate; 0·1 M-sodium allo + DL-isocitrate; 0·05 M-sodium succinate, formate, DL-malate. The reduction of $K_3Fe(CN)_6$ was followed with the Beckman DU spectrophotometer at 400 mμ in 1 cm. cuvettes. Readings were taken at 1 min. intervals for 10 min. Corrections were made for endogenous reduction of the ferricyanide.

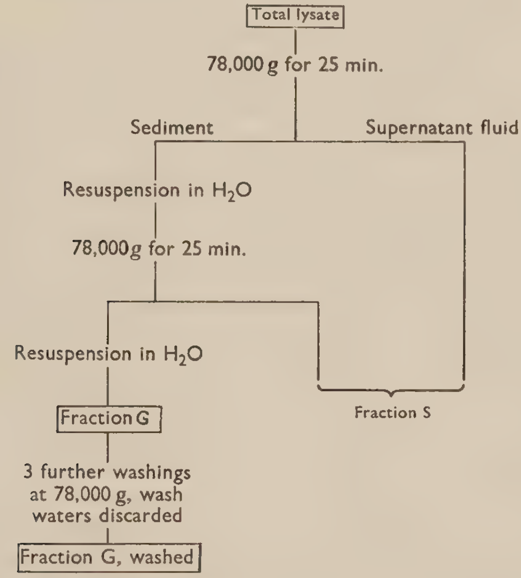
Total lysate
78,000 g for 25 min.
Sediment
Supernatant fluid
Resuspension in H₂O
78,000 g for 25 min.
Resuspension in H₂O
Fraction G
Fraction S
3 further washings at 78,000 g, wash waters discarded
Fraction G, washed

Fig. 1. Centrifugation procedure for the preparation of bacterial fractions.

Fumarase was determined according to the method devised by Racker (1950). The spectrophotometric readings were, however, taken at 305 mμ instead of at 300 mμ since some of the enzyme solutions exhibited a rather strong absorption at the latter wavelength. The reaction mixture contained: 0·5 ml. 0·2 M-phosphate (pH 7·3); 1 ml. 0·05 M neutralized sodium fumarate; 0·5–1·0 ml. enzyme solution; water to 3·0 ml. Readings were taken at 30 sec. intervals for 5 min.

Diphosphopyridine nucleotide (DPNH) oxidase was assayed by measuring the decrease in absorption of the reaction mixture at 340 mμ (Haas, Horecker & Hogness, 1940). The reaction mixture contained: 1·0 ml. 0·05 M-phosphate (pH 6·8); 0·5 ml. 0·001 M-DPNH (obtained from the Sigma Chemical Co.); 0·025–0·050 ml. enzyme solution; water to 3·0 ml. Readings were taken at 30 sec. intervals for 5 min.

Catalase was determined by the permanganate titration method devised by Bonnichsen, Chance & Theorell (1947).

Acid phosphatase was assayed with *p*-nitrophenylphosphate as substrate (Bessey, Lowry & Brock, 1946). The determinations were carried out as described by Boman (1955); 0·100 ml. of enzyme solution was used, however, instead of 0·010 ml., and 0·025 ml. 0·15 M-$MgSO_4$ was added to the reaction mixture as an activating agent. The determinations were carried out at 37°. The pH value of the reaction mixture was 6·0.

Hexokinase was determined by the method described by McDonald (1955). The addition of sodium fluoride did not affect the results. The determinations were carried out at 5°.

With the exception of the decomposition of hydrogen peroxide by catalase, the enzymic reactions studied generally proceeded at constant rates during the whole incubation time.

Chemical analyses. Ribonucleic acid (RNA) was determined according to Schneider (1945), and protein by the biuret method (Gornal, Bardawill & David, 1949; Weibull & Bergström, 1958). Cytochromes were estimated by light absorption measurements. A minute amount of sodium dithionite was added to the suspension or solution to be investigated and the absorption between 500 and 650 mμ was determined with the Beckman model DU spectrophotometer. The amount of cytochromes in the samples was estimated from the height of the peaks at 552, 558 and 600 mμ (when present). A correction for unspecific absorption was made according to Weibull (1948). Material which exhibited strong unspecific absorption was suspended in 70 % (w/v) glycerol instead of water.

The dry weights of bacteria were measured after drying samples at 100° for 24 hr.

Electron microscopy. The electron micrographs were taken with an RCA model EMU-2D electron microscope. The specimens were fixed with osmium tetroxide vapour for 2 hr. at room temperature. The spraying technique devised by Backus & Williams (1950) was used for applying the specimens to the grids. Before fixing and spraying, the specimens were dialysed for 12 hr. against distilled water.

RESULTS

Microscopical morphology of the bacterial extracts

Only one structural element, spherical bodies of low contrast and having a diameter of *c.* 1 μ, was observed in the total lysate and in fraction G ('ghost' fraction) when specimens were viewed under the phase-contrast microscope. In some of these bodies (the 'ghosts'), a few granules were seen. No structures or bodies were seen in fraction S (soluble protoplasm).

Plates 1 and 2, figs. 1–4, show the electron microscopical appearance of sprayed and dried drops of the bacterial fractions. Two kinds of structures can be seen in the total lysate (fig. 1): (i) approximately circular membranes evidently representing 'ghosts'; (ii) small granules forming a coherent mass at the edge of the drops. Practically no small granules are present outside the membranes in fig. 2 ('ghost' fraction) but some granular elements can be seen inside these membranes (cf. Weibull & Bergström, 1958). The membranes are totally missing in fig. 3 (soluble protoplasm). Plate 2, fig. 4, shows fraction S (soluble protoplasm) at a higher magnification and a higher dilution. Both free and aggregated particles can be seen. The smallest particles have a diameter of 100–200 Å. and may thus correspond to the '40 S particles' described by Schachman, Pardee & Stanier (1952) and others. No well-defined elements having larger dimensions than these particles are present in the specimen of soluble protoplasm shown in Pl. 2, fig. 4. Inspection of a large number of similar drops gave the same result.

Table 1. *RNA content of 'ghosts' of* Bacillus megaterium, *strain M*

Each figure represents an average value obtained from three batches of bacteria.

No. of washings of the 'ghosts'	RNA in dried 'ghosts' (%)	RNA in 'ghosts' as % of total RNA
1	1·62	4·6
4	0·82	2·3

Chemical characteristics of the bacterial extracts

Table 1 shows the RNA content of fraction G ('ghost' fraction).

It appears that repeated washing of the 'ghost' fraction decreased its RNA content to some extent. It can also be concluded that at least 95% of the bacterial RNA was present in the soluble protoplasm of *Bacillus megaterium* strain M, since no nucleic acids have been found in the cell wall of this organism (Weibull & Bergström, 1958).

In order to estimate the specific activities of the bacterial enzymes, i.e. activities/mg. protein, the protein content of extracts obtained from three batches of bacteria was determined. According to these analyses, the 'ghost' fraction contained on an average 13% and fraction S (soluble protoplasm) 87% of the protein of the total lysate.

Schachman *et al.* (1952) showed that the bulk of the bacterial RNA in many bacteria is found in particles having a diameter of *c.* 150 Å. and a sedimentation constant of 20–40 S. In order to ascertain to what extent the RNA of *Bacillus megaterium*, strain M, is associated with such particles, fraction S was centrifuged for 2 hr. at 103,000 *g* in a Spinco centrifuge with the no. 40.2 rotor. The supernatant fluid was transferred to fresh tubes and again centrifuged for 2 hr. at 103,000 *g*. Three additional centrifugations of this kind were performed and the supernatants were analysed for RNA. Fig. 2 gives the results. It can be seen that about 15% of the RNA cannot be sedimented at 103,000 *g*. Thus, this part of the bacterial RNA is not associated with particles having a sedimentation constant of 20–40 S.

In order to ascertain whether any sedimentable cytochromes were present in fraction S (soluble protoplasm), this fraction was centrifuged for 20 min. at 103,000 *g*. The sediment was suspended in a small volume of 70% (w/v) glycerol. After adding a minute amount of dithionite the suspension was investigated spectrophotometrically. A peak was observed in the 550–560 mμ range. By comparing the height of this peak with that exhibited by the 'ghost' fraction under similar conditions, it appeared that the cytochromes present in the sedimentable part of the S fraction corresponded to about 5% of the cytochromes of the 'ghost' fraction. When the supernatant fluid of the centrifuged S fraction was investigated spectrophotometrically after a tenfold concentration by pervaporation, no peak at all could be detected in the 500–650 mμ range. It could thus be concluded that the 'ghost' fraction contained about 95% of the cytochromes of the bacterial cells.

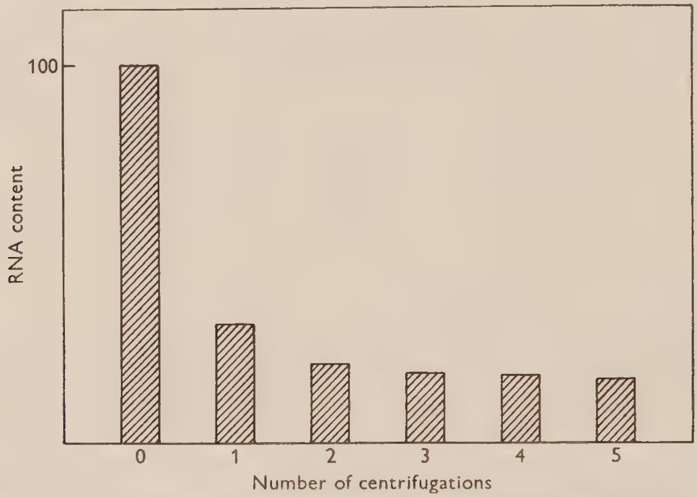

Fig. 2. RNA content of the soluble protoplasm of *Bacillus megaterium*, strain M, before and after centrifugations at 103,000 *g*. Between each centrifugation, the supernatant fluids were transferred to fresh tubes. Relative values are given, the RNA content of the protoplasm before the first centrifugation being put equal to 100.

Enzymic studies

Table 2 shows the results of a series of enzyme determinations carried out on a batch of lysed protoplasts. It can be seen that, for each enzyme, the measured activities were proportional to the amount of enzyme solution used in the tests. Similar results were obtained when fraction G ('ghost' fraction), fraction S (soluble protoplasm) and a reconstituted lysate were tested.

Table 3 shows the amounts of the enzymes studied which were found in the bacterial preparations. Six batches of bacteria were analysed. The activities are expressed as μmole (in the case of catalase mmole) of substrate split, reduced, oxidized or hydrated/min. and/l. of an incubation mixture containing bacterial material equivalent to 1·0 g. (dry wt.) whole bacteria. These figures could easily be calculated from the experimental results since the measured activities were proportional to the amount of enzyme solution used in the tests (see Table 2).

The figures collected in Table 3 shows that the amount of each bacterial enzyme varies considerably from one batch of bacteria to another.

The scope of the present investigation was primarily to study the presence or absence of various enzymes in certain fractions of the bacterial cells. The

Table 2. *The relationship between the enzymic activity of samples of a bacterial lysate (lysed protoplasts of* Bacillus megaterium, *strain M) and the volume of the samples*

Each figure represents one determination. Relative values are given, the activity of each enzyme in a 1 ml. sample being put equal to 100. The ± sign indicates 95 % fiducial limits.

Enzyme tested	Enzymic activity in sample of		
	1 ml.	0·5 ml.	0·25 ml.
Succinic dehydrogenase	100	51·1	23·2
Malic dehydrogenase	100	46·0	23·8
Lactic dehydrogenase	100	46·8	26·7
Isocitric dehydrogenase	100	53·9	23·7
Fumarase	100	53·1	26·9
DPNH oxidase	100	44·0	24·0
Catalase	100	52·3	27·8
Hexokinase	100	52·0	—
Acid phosphatase	100	49·1	24·0
Average	100	49·8 ± 2·7	25·0 ± 1·5

Table 3. *Enzymic activities exhibited by six different batches of* Bacillus megaterium, *strain M*

The minimum and maximum values found are given in the table. The enzymic activities are expressed as μmole (in the case of catalase, mmole) of substrate split, reduced, oxidized or hydrated per min. and per l. of an incubation mixture containing material equivalent to 1 g. dry wt. bacteria. In the case of the catalase-hydrogen peroxide reaction, the initial decomposition rate was used for calculating the enzymic activity. All other reactions proceeded generally at constant rates during the incubation time.

Enzyme tested	Total lysate	Fraction G ('ghost' fraction)	Fraction S (soluble protoplasm)	Reconstituted lysate
Succinic dehydrogenase	0·80–2·03	1·24–3·06	< 0·02–0·23	0·67–2·75
Malic dehydrogenase	3·19–5·55	0·80–2·80	0·61–0·81	1·31–3·15
Lactic dehydrogenase	0·87–2·03	0·41–0·92	0·56–1·21	0·93–2·10
Isocitric dehydrogenase	0·62–0·90	< 0·02–0·12	0·73–1·06	0·03–0·37
Formic dehydrogenase	< 0·02	< 0·02	< 0·02	< 0·02
DPNH oxidase	5·58–25·2	19·6–46·5	< 0·2	10·6–37·2
Fumarase	145–376	33·6–194	138–348	105–326
Catalase	4·85–17·0	0·06–0·22	4·60–18·2	5·30–15·2
Hexokinase	3·56–10·9	0·40–0·53	3·00–10·2	4·56–14·2
Acid phosphatase	0·78–1·65	0·01–0·06	0·79–1·97	0·59–1·48

situation in this respect is most clearly demonstrated when the relative amounts of each enzyme in the fractions studied are compared with one another. For this purpose, the enzyme content of each bacterial fraction was calculated, giving the amount of each enzyme in the total bacterial lysate a value of 100. Table 4 shows the results; each figure is the average of determinations carried out on six batches of bacteria. Three of the investigated 'ghosts' fractions were washed once, the remainder four times (see Fig. 1). No significant differences between the enzymic activities of these two types of ghosts were observed.

It can be seen from the figures in Table 4 that less than 10 % of the succinic dehydrogenase and the DPNH oxidase of the lysed bacteria were found in fraction S (soluble protoplasm). The bulk of these enzymes were found in the 'ghost' fraction. On the other hand, less than 10% of the isocitric dehydrogenase, the catalase, the hexokinase and the acid phosphatase were found in the 'ghost' fraction. Considerable amounts of lactic and malic dehydrogenase and of fumarase were present in both fraction S and the 'ghost' fraction. The recoveries of malic and isocitric dehydrogenase in the reconstituted lysate were low, whereas the DPNH-oxidase activity was considerably higher in this system than in the original lysate. The DPNH oxidase activity of the 'ghosts' was also remarkably high.

Table 4. *Relative amounts of enzymes in cell extracts prepared from* Bacillus megaterium, *strain M*

The average values obtained from six batches of bacteria are given. The activity of each enzyme in the total lysate is put equal to 100. The bacterial material present in each extract was derived from the same amount of bacteria. The ± sign indicates 95% fiducial limits.

Enzyme	Total lysate	Fraction G ('ghost' fraction)	Fraction S (soluble protoplasm)	Reconstituted lysate
Succinic dehydrogenase	100	145·3 ± 21·1	4·5 ± 4·7	136·8 ± 18·9
Malic dehydrogenase	100	32·6 ± 16·3	15·7 ± 2·9	49·7 ± 14·5
Lactic dehydrogenase	100	41·2 ± 10·2	57·2 ± 11·2	102·8 ± 15·4
Isocitric dehydrogenase	100	3·5 ± 6·4	112·0 ± 19·4	27·1 ± 15·3
Fumarase	100	32·2 ± 11·8	101·8 ± 17·9	112·8 ± 22·1
DPNH oxidase	100	261·5 ± 61·0	< 1	209·0 ± 76·0
Catalase	100	1·3 ± 0·7	100·2 ± 11·9	92·0 ± 3·6
Hexokinase	100	5·5 ± 2·0	87·5 ± 8·7	143·4 ± 22·7
Acid phosphatase	100	3·0 ± 1·6	99·6 ± 16·1	83·6 ± 8·9

As mentioned in the preceding section, the protein content of the 'ghosts' was 0·13 and that of fraction S (soluble protoplasm) 0·87 when the protein content of the total lysate was put equal to 1·00. These values were used for calculating the concentration of the enzymes, i.e. the enzymic activities/g. protein. Table 5 shows the results. The specific activity of each enzyme in the total lysate was put equal to 1·00.

It was shown above that about 85% of the bacterial RNA was sedimented at 103,000 *g*. It was considered of interest to establish whether some of the enzymes found in the S fraction (soluble protoplasm) could be sedimented in the same field. For this purpose samples of the S fraction were centrifuged for 2 × 2 hr. at 103,000 *g* transferring the supernatant fluids to fresh tubes between the centrifugations. The RNA content and the enzymic activities of the supernatant fluid were then determined. The results are given in Table 6 from which it can be seen that, on the average, about 85% of the various enzymic activities remained in the supernatant fluid whereas about 78% of the RNA was sedimented. Since about 15% of the total RNA was not sedimented even after repeated centrifugations (see Fig. 2), it can be concluded that more than 90% of the sedimentable RNA present in the bacteria can be removed from the protoplasm without affecting seriously the activities of the enzymes studied.

Table 5. *Concentration of enzymes (enzymic activities/g. protein) in fractions prepared from* Bacillus megaterium, *strain M*

The average values obtained from six batches of bacteria are given. The concentration of each enzyme in the total lysate is put equal to 1·00. The ± sign indicates 95 % fiducial limits.

Enzyme	Total lysate	Fraction G ('ghost' fraction)	Fraction S (soluble protoplasm)
Succinic dehydrogenase	1·00	11·2 ± 1·6	0·05 ± 0·05
Malic dehydrogenase	1·00	2·51 ± 1·25	0·18 ± 0·03
Lactic dehydrogenase	1·00	3·17 ± 0·79	0·66 ± 0·13
Isocitric dehydrogenase	1·00	0·27 ± 0·49	1·29 ± 0·22
Fumarase	1·00	2·48 ± 0·91	1·17 ± 0·21
DPNH oxidase	1·00	20·1 ± 4·7	< 0·01
Catalase	1·00	0·10 ± 0·05	1·15 ± 0·14
Hexokinase	1·00	0·42 ± 0·15	1·01 ± 0·10
Acid phosphatase	1·00	0·23 ± 0·12	1·14 ± 0·18

Table 6. *RNA content and enzymic activities of the soluble protoplasm of* Bacillus megaterium, *strain M, before and after centrifugation at* 103,000 *g for* 2 × 2 *hr.*

Between the centrifugations, the supernatant fluids were transferred to fresh tubes. Relative values are given, the enzymic activities and the RNA content before the first centrifugation being put equal to 100. The ± sign indicates 95 % fiducial limits.

Enzyme	Activity after centrifugation, % of initial values
Malic dehydrogenase	74·4 ± 8·4
Isocitric dehydrogenase	91·5 ± 11·4
Fumarase	83·0 ± 6·5
Hexokinase	76·8 ± 9·3
Acid phosphatase	88·8 ± 9·0
Catalase	103·8 ± 18·4
RNA	22·6 ± 1·6

DISCUSSION

The aim of the present investigation was primarily to locate certain enzymes or more precisely certain enzymically-active proteins in *Bacillus megaterium*, strain M. The method used, fractionation of lysed organisms by differential centrifugation and determination of enzymic activities in the fractions, is, like other methods used for the same purpose, subject to several sources of error. First, the subcellular structures to be investigated may be more or less degraded during the lysis, secondary aggregates may be formed and adsorption phenomena may take place. Secondly, the lysis and the fractionation procedure may involve the denaturation of enzyme proteins. Thirdly, inhibitory and activating agents may influence the enzymes studied to different degrees in the various bacterial fractions. This is especially likely when the enzymes are not purified before their activities are measured.

To avoid misinterpretations of the experimental results, we have taken into consideration the criteria proposed by Hogeboom, Schneider & Striebich (1953) and by Alexander & Wilson (1955). These criteria can be formulated as follows (cf. Alexander & Wilson, 1955). First, for each enzyme, the sum of the activities of the isolated fractions should approach 100 % of that found in the original extract. Secondly, a large percentage of the total activity should reside in the fraction to which the enzymic function is being attributed. Thirdly, the concentrations of the various enzymes (i.e. enzyme activities/g. protein) in the isolated fractions should be similar to or higher than those in the total lysate.

When the first two of these criteria are taken into account, the figures of Table 4 evidently strongly suggest that the succinic dehydrogenase and the DPNH oxidase of *Bacillus megaterium*, strain M, are located in the bacterial 'ghosts'. The isocitric dehydrogenase, the catalase, the hexokinase and the acid phosphatase, on the other hand, seem to be situated in the soluble protoplasm. No certain conclusions can be drawn about the location of the malic and lactic dehydrogenases and the fumarase.

When the figures in Table 5 are taken into account, then the third criterion can be used for testing the conclusions drawn in the preceding paragraph. In the main, these conclusions are not affected. It is evident, however, that the concentrations of the malic and lactic dehydrogenases are appreciably higher in the proteinaceous part of the 'ghosts' than in the same part of the soluble protoplasm. The concentration of the malic dehydrogenase in the soluble protoplasm is remarkably low. However, the total recovery of the malic dehydrogenase is also low (see Table 4).

The activities of the DPNH oxidase and the succinic dehydrogenase are appreciably higher in the sum of the fractions than in the total lysate. This might be due to an 'unmasking' effect (Alexander, 1956). It is more difficult to explain the low activities of the malic and the isocitric dehydrogenases in the reconstituted lysate.

A comparison between our results and those obtained by Storck & Wachsman (1957) suggests that the enzymic organization of cells of the KM strain of *Bacillus megaterium* is rather similar to that of the M strain. A difference may be indicated by the fact that Storck & Wachsman found lactic dehydrogenase mainly in the 'ghosts' from the KM strain, while we found this enzyme in the 'ghosts' and in the soluble protoplasm of the M strain. Both investigations indicate, however, a high specific activity for the 'ghosts'.

With respect to other bacterial species, the present investigation confirms earlier results concerning the location of the cytochrome system, the DPNH oxidase, the succinic dehydrogenase and the isocitric dehydrogenase (Alexander, 1956; Mitchell & Moyle, 1956*b*; Cota-Robles, Marr & Nilson, 1958). Mitchell & Moyle (1956*b*) found malic and lactic dehydrogenases mainly in the plasma-membrane fraction of *Staphylococcus aureus*. Linnane & Still (1955), however, found the same enzymes both in the particulate and soluble fractions of the protoplasm of *Serratia marcescens*. According to Alexander (1956) fumarase is situated mainly in the soluble protoplasm of several bacterial species.

Our findings concerning the location of catalase agree with the experiments of Few, Fraser & Gilby (1957). These workers found that the catalase of intact *Micrococcus lysodeikticus* cells was confined to the interior of the organisms since the activity of this enzyme was independent of the pH value of the surrounding medium. On the other hand, Alexander & Wilson (1955) reported that catalase was present in all fractions of the protoplasm of *Azotobacter vinelandii* which they examined.

Contrary to our results, Mitchell & Moyle (1956*b*) found acid phosphatase mainly in the plasma-membrane fraction of *Staphylococcus aureus.* To our knowledge, no investigations have been made on the distribution of hexokinase in bacteria. According to our experiments, the hexokinase, like the acid phosphatase, is probably not present in the 'ghosts' and hence not in the cytoplasmic membrane of *Bacillus megaterium*, strain M. This would imply that these enzymes are not directly involved in the transportation of glucose and phosphatase across the permeability barrier of the cells (cf. Rothstein, 1954; Mitchell, 1957).

The enzymic properties of those particles of the bacterial cytoplasm which have a sedimentation constant of about 40 S and which contain the bulk of the bacterial RNA have been discussed repeatedly (Schachman *et al.* 1952; Bradfield, 1956; Alexander, 1956). None of the enzymes studied by us was found to be associated with such particles, since these enzymes were located either in the bacterial 'ghosts', which contain only 2–4 % of the bacterial RNA, or in the soluble protoplasm, from which more than 90 % of the sedimentable RNA could be removed without seriously affecting the enzymic activities. In this connexion it should be mentioned that, according to a recent report (Cota-Robles *et al.* 1958), no enzymes except a ribonuclease were found in the RNA-containing particles of *Azotobacter agilis.*

If one assumes that at least some enzymes exist exclusively in certain subcellular structures of the bacterial cell, then the purity of the fractions isolated by us could be estimated from the enzymic determinations. Thus fraction S (soluble protoplasm) may contain about 5 % of 'ghost' material (according to the electron microscopical findings in a highly fragmented form) since 5 % of the total succinic dehydrogenase of the lysed cells was found in this fraction. The same conclusion can be drawn from the spectrophotometric cytochrome determinations. Conversely, about 5 % of the soluble protoplasm may be adsorbed on the 'ghosts', judging from the amounts of isocitric dehydrogenase, catalase, hexokinase and acid phosphatase found in the 'ghost' fraction.

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REFERENCES

ALEXANDER, M. (1956). Localization of enzymes in the microbial cell. *Bact. Rev.* **20**, 67.

ALEXANDER, M. & WILSON, P. W. (1955). Enzyme localization in *Azotobacter vinelandii*. *Proc. nat. Acad. Sci., Wash.* **41**, 843.

BACKUS, R. C. & WILLIAMS, R. C. (1950). The use of spraying methods and of volatile suspending media in the preparation of specimens for electron microscopy. *J. appl. Phys.* **21**, 11.

BAUMANN-GRACE, J. B. & TOMCSIK, J. (1957). The surface structure and serological typing of *Bacillus megaterium*. *J. gen. Microbiol.* **17**, 227.

BESSEY, O. A., LOWRY, O. H. & BROCK, M. J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. biol. Chem.* **164**, 321.

BOMAN, H. G. (1955). Chromatography of prostatic phosphatase. *Biochim. biophys. Acta*, **16**, 245.

BONNICHSEN, R. K., CHANCE, B. & THEORELL, H. (1947). Catalase activity. *Acta chem. scand.* **1**, 685.

BRADFIELD, J. R. C. (1956). Organization of bacterial cytoplasm. In *Bacterial Anatomy, Symp. Soc. gen. Microbiol.* **6**, 296.

COTA-ROBLES, E. H., MARR, A. G. & NILSON, E. H. (1958). Submicroscopic particles in extracts of *Azotobacter agilis*. *J. Bact.* **75**, 243.

FEW, A. V., FRASER, M. J. & GILBY, A. R. (1957). The intracellular catalase of *Micrococcus lysodeikticus*. *Biochim. biophys. Acta*, **24**, 306.

GILBY, A. R., FEW, A. V. & MCQUILLEN, K. (1958). The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus*. *Biochim. biophys. Acta*, **29**, 21.

GLADSTONE, G. P. & FILDES, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751.

HAAS, E., HORECKER, B. L. & HOGNESS, T. R. (1940). The enzymatic reduction of cytochrome C. *J. biol. Chem.* **136**, 747.

HOGEBOOM, G. H., SCHNEIDER, W. C. & STRIEBICH, M. J. (1953). Localization and integration of cellular function. *Cancer Res.* **13**, 617.

LINNANE, A. W. & STILL, J. L. (1955). The intracellular distribution of enzymes in *Serratia marcescens*. *Biochim. biophys. Acta*, **16**, 305.

MCDONALD, M. R. (1955). *Methods in Enzymology*. Ed. by Colowick, S. P. & Kaplan, N. O., I, p. 269. New York: Academic Press. Inc.

MARR, A. G. & COTA-ROBLES, E. H. (1957). Sonic disruption of *Azotobacter vinelandii*. *J. Bact.* **74**, 79.

MITCHELL, P. (1957). A general theory of membrane transport from studies of bacteria. *Nature, Lond.* **180**, 134.

MITCHELL, P. & MOYLE, J. (1956*a*). Osmotic function and structure in bacteria. In *Bacterial Anatomy, Symp. Soc. gen. Microbiol.* **6**, 150.

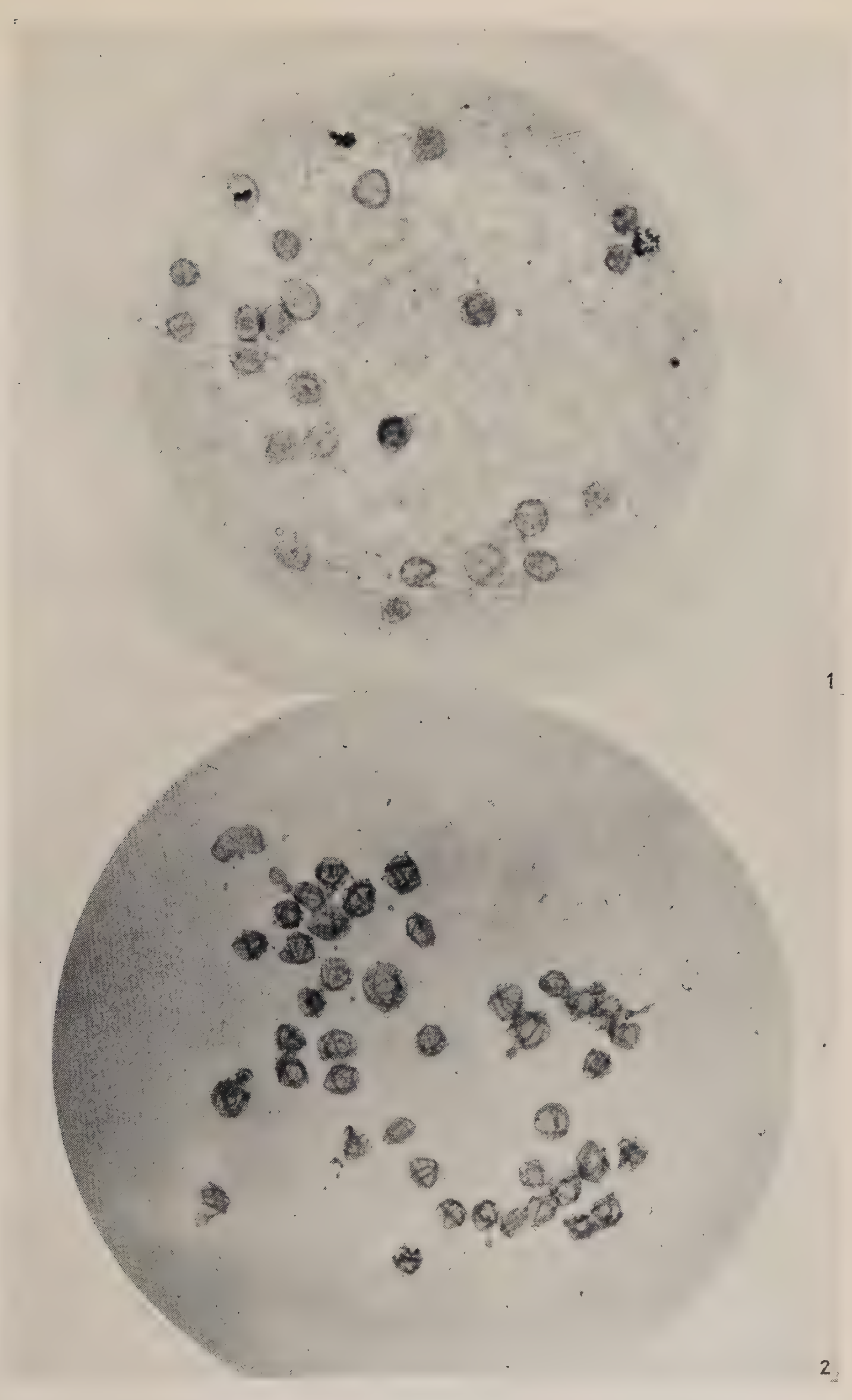
MITCHELL, P. & MOYLE, J. (1956*b*). The cytochrome system in the plasma membrane of *Staphylococcus aureus*. *Biochem. J.* **64**, 19P.

RACKER, E. (1950). Spectrophotometric measurement of the enzymatic formation of fumaric and *cis*-aconitic acids. *Biochim. biophys. Acta*, **4**, 211.

ROTHSTEIN, A. (1954). The enzymology of the cell surface. In *Protoplasmatologia*. Ed. by Heilbrunn, L. V. & Weber, F. II, E4. Wien, Innsbruck: Springer-Verlag.

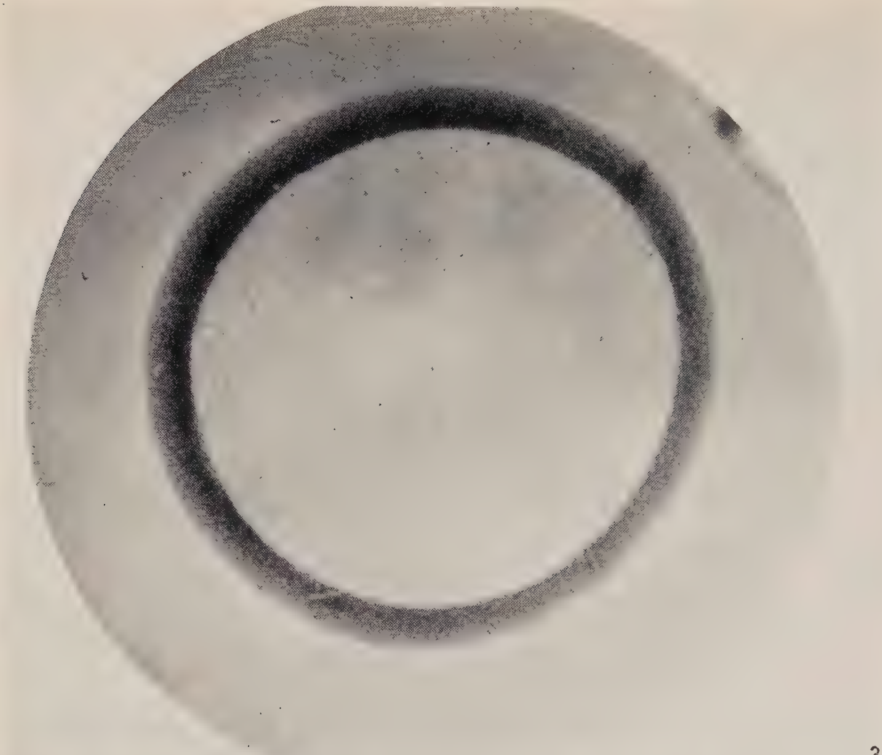
SCHACHMAN, H. K., PARDEE, A. B. & STANIER, R. Y. (1952). Studies on the macromolecular organization of microbial cells. *Arch. Biochem. Biophys.* **38**, 245.

SCHNEIDER, W. C. (1945). Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.



C. Weibull, H. Beckman & L. Bergström—Enzymes in *Bacillus megaterium*.
Plate 1

(*Facing p.* 530)



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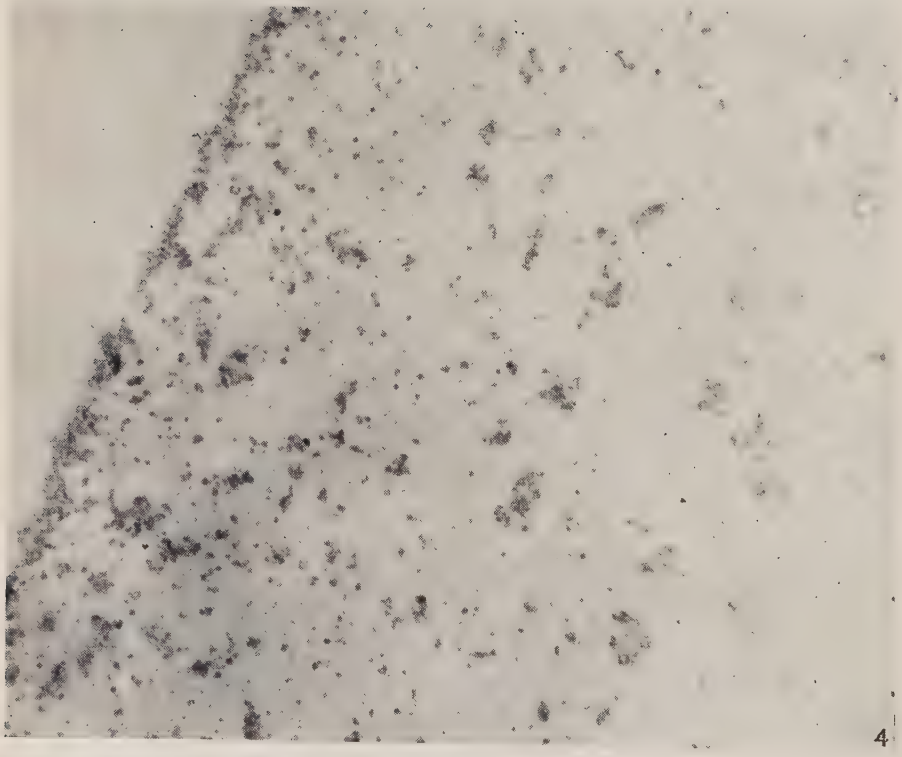

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C. WEIBULL, H. BECKMAN & L. BERGSTRÖM—ENZYMES IN *BACILLUS MEGATERIUM.* PLATE 2

Spiegelman, S., Aronson, A. I. & Fitz-James, P. C. (1958). Isolation and characterization of nuclear bodies from protoplasts of *Bacillus megaterium*. *J. Bact.* **75**, 102.

Storck, R. & Wachsman, J. T. (1957). Enzyme localization in *Bacillus megaterium*. *J. Bact.* **73**, 784.

Vennes, J. W. & Gerhardt, P. (1956). Immunological comparison of isolated surface membranes of *Bacillus megaterium*. *Science*, **124**, 535.

Weibull, C. (1948). Some chemical and physico-chemical properties of the flagella of *Proteus vulgaris*. *Biochim. biophys. Acta*, **2**, 351.

Weibull, C. (1953*a*). The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. *J. Bact.* **66**, 688.

Weibull, C. (1953*b*). Characterization of the protoplasmic constituents of *Bacillus megaterium*. *J. Bact.* **66**, 696.

Weibull, C. (1956). The nature of the 'ghosts' obtained by lysozyme lysis of *Bacillus megaterium*. *Exp. Cell. Res.* **10**, 214.

Weibull, C. (1957). The lipids of a lysozyme sensitive *Bacillus* species (*Bacillus* 'M'). *Acta chem. scand.* **11**, 881.

Weibull, C. & Bergström, L. (1958). The chemical nature of the cytoplasmic membrane and cell wall of *Bacillus megaterium*, strain M. *Biochim. biophys. Acta*, **30**, 340.

EXPLANATION OF PLATES

Plates 1–2

Electron micrographs of sprayed drops of bacterial extracts obtained by shocking osmotically protoplasts of *Bacillus megaterium*, strain M. The extracts were dialysed for 12 hr. against distilled water and fixed for 2 hr. at room temperature with osmium tetroxide vapour.

Plate 1

Fig. 1. Total lysate. × 3000.
Fig. 2. Fraction G ('ghost' fraction). × 3000.

Plate 2

Fig. 3. Fraction S (soluble protoplasm). × 3000.
Fig. 4. Fraction S (diluted 1/100). × 32,000.

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Brown, A. M. (1959). *J. gen. Microbiol.* **20**, 532–539

Growth Idiosyncrasies of Some *Bordetella pertussis* Strains, and the Intracerebral Virulence of these Strains in Mice

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SUMMARY: The growth characteristics of eight strains of *Bordetella pertussis* were studied in defined liquid media. Two of these strains (1017, 1018), which are both very virulent on intracerebral injection into mice, were found to have growth idiosyncrasies. Both these strains differed from typical virulent pertussis strains and from a degraded strain in that they needed more magnesium and a different gas phase (carbon dioxide and oxygen) for optimal growth in defined liquid medium. Both strains were inhibited by crystalline lysozyme (100 mg./ml.) in defined medium, whereas typical virulent strains and a degraded strain were insensitive to it.

The growth of *Bordetella pertussis* was reviewed by Rowatt (1957) who concluded that 'knowledge of the metabolism of these organisms is slight'. Conditions for their growth on solid media and in defined liquid media were discussed, but no reference was made to growth idiosyncrasies of strains which were highly virulent when injected intracerebrally into mice. The study of the intracerebral invasion in mice of two strains of *B. pertussis* (Brown, 1958) led to the conclusion that the shape of the growth curves obtained was determined by the balance between the growth in, and removal of the organisms from, the brain, and was characteristic for each strain. The following work is a study of the growth of some *B. pertussis* strains of determined intracerebral virulence in defined liquid media.

METHODS

Organisms. Eight strains of *Bordetella pertussis* were used: five smooth, virulent, freshly isolated strains (1011 (NCTC 7234), 1012 (NCTC 20948), 1013 (NCTC 21194) and 1014, 1015 from Dr B. W. Lacey), two smooth virulent strains (1017 described by Kendrick, Eldering, Dixon & Misner, 1947, 1018 described by Andersen 1952; both were highly virulent when injected by the intracerebral route). One degraded strain 1011 HR derived from strain 1011 by continuous subculture on Bordet-Gengou medium made with 50% (v/v) horse blood.

Measurement of virulence of strains. The LD 50 dose for each strain determined in albino male Swiss bred mice weighing 17–20 g. in the manner described previously (Brown, 1958) was considered the measure of its virulence. Values obtained both by intracerebral injection and by intranasal instillation were the average of at least two results.

Plate counts of pertussis organisms. These were made by the method of

Miles & Misra (1938). No penicillin was added to the medium as knowledge of the presence of contaminants was essential.

Preparation of suspensions of organisms for the inoculation of growth experiments. The storage of organisms, and their recovery from the dried state for the preparation of suspensions, was as previously described (Brown, 1958). In this work all organisms were washed once with saline and resuspended in the medium described by Jebb & Tomlinson (1955). The estimation of total numbers in each suspension was made by opacity measurements as before. Dilutions of suspensions which contained approximately 4×10^9 viable organisms/ml. were prepared, and unless otherwise stated the inoculation of cultures in growth experiments was approximately 4×10^3 organisms/ml. medium.

Defined liquid medium and growth experiments

Basal medium. The medium described by Jebb & Tomlinson (1955) was found satisfactory for this work. It was essential to boil the medium during charcoal absorption or to stir efficiently when the process was done in the steamer. Uneven distribution of the charcoal during absorption produced a medium which did not appear to have a uniform composition. Nicotinamide (to 4×10^{-7} M) and haemin (1 mg./ml.; added as haematin) were the growth factors added. Magnesium sulphate was substituted for magnesium chloride in the medium.

Design of growth experiments. Volumes (20 ml.) of basal medium were dispensed in 100 ml. hard glass square bottles which were closed with loose aluminium caps, and autoclaved for 10 min. at 15 lb./sq.in. Additions of nicotinamide, haematin and any other substances were made under aseptic conditions immediately before inoculation. Two bottles of each variant of the medium were inoculated with every test strain of organism. The cultures were incubated on racks inclined at an angle of 45° in a hot room at 36°. Illumination from behind with a strip light enabled the course of growth to be readily followed. Experiments of interest were repeated with all strains and more accurate appraisal of the growth of some strains was made by colony counts. These counts were the average of counts obtained from the duplicate cultures. The final amount of growth, when required, was estimated with a Hilger Biochem Absorptiometer. Maximum yields of organisms from the medium was not sought, and no extra glutamate was added to give this.

In two experiments cultures from some strains were shaken on a Kahn shaker (Townson & Mercer). This has a horizontal stroke of 4 cm. and was shaken at approximately 120 strokes/min. The rheostat was removed from the body of the shaker and the tray was insulated with rubber to prevent overheating of the cultures. These experiments are more fully described below.

RESULTS

Virulence of test strains. The approximate LD 50 doses for the eight strains are given in Table 1. No mice were killed by strain 1011 HR, at the maximum dose tested (20×10^6 organisms) so this strain is called avirulent. The results for strains 1017 and 1018 which showed an intracerebral LD 50 of 400 and 145 organisms, respectively, indicate the considerable intracerebral virulence of these strains, while the remaining strains all showed some virulence whether given by the intracerebral or intranasal routes.

Table 1. *Approximate* LD 50 *doses for strains of* Bordetella pertussis

Strain	Intra-cerebral LD 50 (in millions of organisms)	Intra-nasal LD 50 (in millions of organisms)
1011	0·2	0·20
1012	33·0	0·36
1013	10·9	1·10
1014	1·1	—
1015	5·4	0·43
1017	0·0004	6·00
1018	0·0015	0·45
1011 HR	> 20·0	> 20·00

Growth experiments

Growth of test strains of pertussis organisms in basal media plus increasing quantities of magnesium sulphate. Each of the test strains was grown in basal medium containing respectively 0·048, 0·096, 0·24, 0·48 and 0·96 mg. $MgSO_47H_2O$/ml. Visual examination of cultures showed that strains 1017 and 1018 grew more slowly with smaller quantities of magnesium sulphate than strains 1011 and 1015 and the avirulent strain 1011 HR. This was confirmed by counts of the growth of strains 1015, 1017, 1018 and 1011 HR in media containing, respectively, 0·048 and 0·96 mg. $MgSO_47H_2O$/ml. Strain 1015 was representative of the strains 1011 to 1015. The results are given in Fig. 1 and show the striking improvement in growth of strains 1017 and 1018 with the larger amount of magnesium sulphate. Magnesium sulphate was therefore used in all following experiments to augment the basal medium.

Growth of test strains of pertussis organisms in basal media at pH *values* 7·3–7·9 *and augmented by* $MgSO_47H_2O$ to 0·48 mg./ml. Rowatt (1957) found that pertussis organisms showed little variation in growth with the pH value of the medium. This was confirmed for most of the test strains, 1017 being the exception (see Fig. 2). The final amounts of growth obtained differed, some strains (e.g. 1015) giving a greater yield in a medium at a lower pH value, and some (e.g. 1018) a lesser yield.

A further experiment in which cultures of strains 1011, 1017 and 1018 were incubated on the shaker gave the results in Figs. 3 and 4. The growth of organisms obtained in 2 days from strain 1011 was considerably less from media at pH 7·3 than from media at pH 7·86, whilst not only was no growth obtained from strain 1017 in shaken culture but the death of the organisms was more rapid at pH 7·3 than at pH 7·86. Final growth determinations made by opacity measurements for all these strains are quoted in Table 2. They show the inability of strain 1017 to grow and the poor growth obtained from

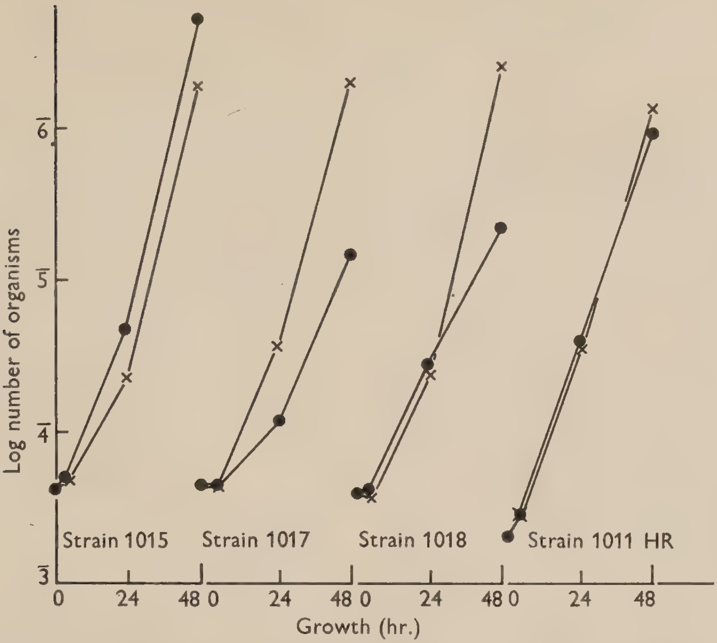

Fig. 1. Effect of concentration of magnesium sulphate on growth of four strains of *Bordetella pertussis* in augmented Jebb & Tomlinson medium. ●—●, 0·048 mg. $MgSO_47H_2O$/ml.; ×—×, 0·96 mg. $MgSO_47H_2O$/ml.

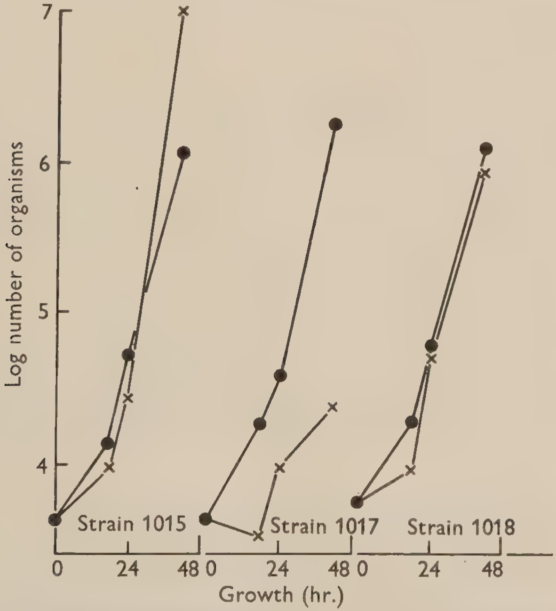

Fig. 2. Growth of *Bordetella pertussis* strains 1015, 1017 and 1018 at pH 7·86 and 7·3. ●—●, growth at pH 7·86; ×—×, growth at pH 7·3.

strain 1018 when 20 ml. cultures were shaken in 100 ml. containers, whatever the pH value of the medium.

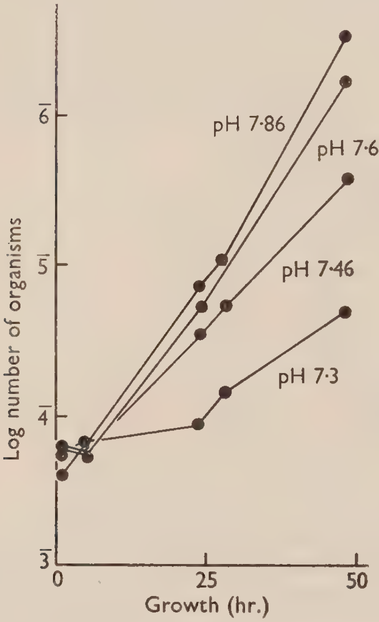

Fig. 3

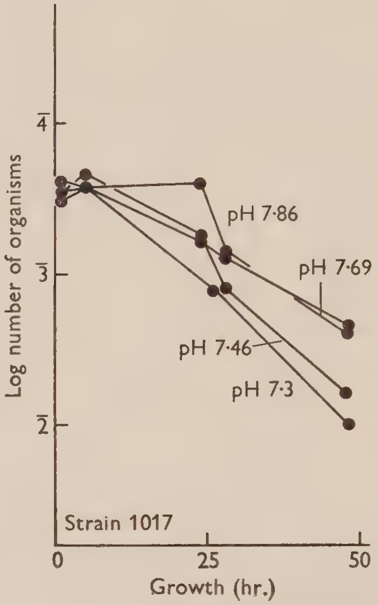

Fig. 4

Fig. 3. Growth of *Bordetella pertussis* strain 1011 on shaking machine in media at different pH values.

Fig. 4. Growth of *Bordetella pertussis* strain 1017 on shaking machine in media at different pH values.

Table 2. *Measurement by % absorption of light of final growth of* Bordetella pertussis *strains* 1011, 1017 *and* 1018 *in* 20 *ml. volumes of media at different* pH *values*

The cultures were incubated at 36° on a shaker in 100 ml. bottles.

	pH of media			
	7·86	7·69	7·46	7·3
Strains	% absorption of light			
1011	85·5	86·2	83·5	81·5
1017	No absorption—no growth			
1018	1·0	62·4	28·4	27·0

Growth of four of the strains of pertussis organisms in basal medium containing catalase (1 *unit/ml.*) *to replace the usual content of haematin.* Two 20 ml., two 40 ml. and two 60 ml. volumes of medium were inoculated with each of strains 1011, 1012, 1017 and 1018, and these cultures were incubated at 36° on the shaker. In this experiment catalase, which was found by Mazloum &

Rowley (1955) to initiate the growth of pertussis organisms on solidified Cohen & Wheeler medium (1946), was used to replace the haematin usually added. Growth appeared first in the 60 ml. volumes of medium for all strains. Slight delay only occurred in 40 ml. of medium, and strains 1011 and 1012 grew almost equally as well in 20 ml. medium. There was, however, no growth of strain 1017 in 20 ml. of medium and only slight growth in one of the 20 ml. cultures inoculated with strain 1018. This experiment was repeated with virtually the same results. It must be concluded that the conditions which obtain when 20 ml. of culture are shaken in a 100 ml. square bottle do not allow optimal growth of any of these strains, but that the growth of strains 1017 and 1018 is more sensitive to a different gas phase composition of carbon dioxide and oxygen than the other strains examined.

The growth of pertussis organisms in a control medium compared with that in a similar broth to which crystalline lysozyme has been added. The growth from eight strains of pertussis organisms in the basal medium augmented with 0·48 mg. $MgSO_47H_2O$/ml. at *c.* pH 7·8 was compared with that in the same medium to which crystalline lysozyme had been added. The concentration of lysozyme used in the experiment (100 mg./ml.) was approximately that present in horse sera; mouse sera contained a similar concentration, but sheep sera only *c.* one-tenth the amount. Lungs washed with saline, macerated in 10 ml. saline and the supernatant fluid titrated for lysozyme were found to contain *c.* 1000 mg. lysozyme/lung. Brains treated in the same way appeared to contain little. All these titrations were made with *Micrococcus lysodeikticus* in conjunction with crystalline lysozyme (Light & Co.).

Counts of only four strains were made and those in the control medium are not shown as the growth of all the strains was very similar. The counts of organisms from strains 1011 and 1012 (Fig. 5) illustrate the growth of six of the test strains in the presence of lysozyme. The inhibition of the growth of strains 1017 and 1018 is shown by the decreasing number of organisms with these strains. The possibility that this difference in reaction might be due to an impurity in the lysozyme must be considered. If 1 % of such an impurity were present, it would need to inhibit strains 1017 and 1018 in a concentration of 1 mg./ml. of medium. This possibility has not been investigated.

The partial reversal of the inhibition by lysozyme on the growth of pertussis organisms. The growth of four strains of pertussis organisms (1011, 1013, 1015 and 1017) in Jebb & Tomlinson medium containing added yeast dialysate instead of nicotinamide was compared with that in a similar medium to which 100 mg. lysozyme/ml. had been added. Inoculation with approximately 10^3 organisms of each strain was made into the control medium and into medium containing lysozyme. Weekly and twice weekly subcultures were made with a platinum loop. All strains continued to grow for 16 and 32 subcultures, respectively, in both media, although visible growth of strain 1017 was somewhat slower from the original inoculum. The inhibition noted in the previous experiment in the presence of lysozyme must have been annulled by some factor in the yeast dialysate.

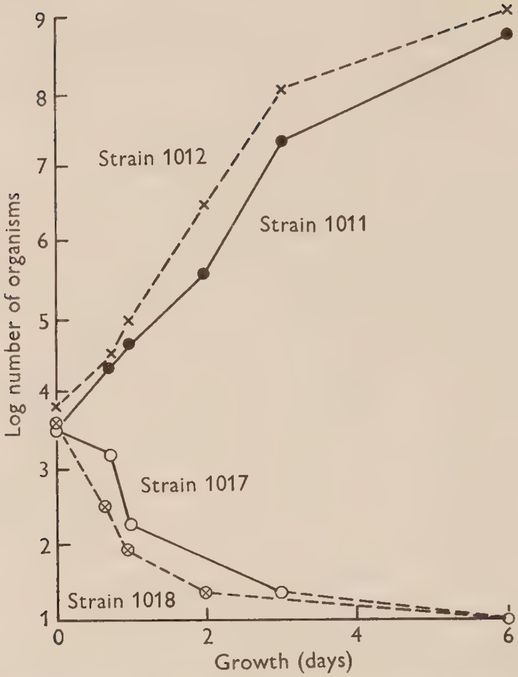

Fig. 5. Counts of organisms from growth of *Bordetella pertussis* strains in augmented Jebb & Tomlinson medium + 100 mg. lysozyme/ml.

DISCUSSION

The idiosyncrasies of growth described in the previous experiments are restricted to *Bordetella pertussis*, strains 1017 and 1018. These strains have LD 50 of 400 and 1500 organisms, respectively, on intracerebral injection, values at least a thousand times smaller than the LD 50 doses of the other test strains. Lacey (1951) showed that *B. pertussis* grown on solid medium containing 33 % (v/v) horse blood and magnesium sulphate instead of sodium chloride in the agar base, changed in antigenic structure. In the present work it has been shown that strains 1017 and 1018, which are very virulent when injected into mice by the intracerebral route, need more magnesium for optimal growth in the defined medium than the less virulent or avirulent strains examined. I did not examine these strains for antigenic differences from typical virulent strains. However, the avirulent degraded strain, which would be expected to differ antigenically from typical virulent strains, did not differ in its magnesium requirements from the typical virulent strains.

The inhibition of the growth of strains 1017 and 1018 by lysozyme in this particular basal medium was repeated regularly. The possibility that the inhibition is due to an impurity has not been explored, but when yeast dialysate was used as growth factor instead of nicotinamide growth was only delayed. *Bordetella pertussis*, strains 1011 and 1017, suspended in 1 % saline containing 0·1 % sodium bicarbonate were not lysed by 1 mg. lysozyme/ml., as determined by opacity measurements with the Hilger Biochem Absorptiometer. Also I failed to detect glucosamine or acetylglucosamine in the supernatant fluids from a similar suspension of strain 1011 incubated with lysozyme. It is clear that lysozyme is present in all Bordet-Gengou media (in the horse blood) in which pertussis organisms grow well. Lysozyme is also widely distributed in body tissues and fluids, although I failed to find it in mouse brains in which strains 1017 and 1018 grow so well from small inocula. Certain surface active substances, e.g. polymyxin, otherwise 'aerosporin' (Ainsworth, Brown & Brownlee, 1947) and 'Tween 80' inhibit growth of typical pertussis organisms *in vitro*. No surface acting substance, however, has been noted which reveals a difference between those strains which are very virulent on intracerebral injection and those which are normally virulent strains. These *in vitro* properties are not necessarily connected with the difference in the rate of growth noted in mouse brains for strains 1011 and 1017 (Brown, 1958). It is, however, interesting that those strains more virulent when injected into mice by the intracerebral route should show the idiosyncrasies of growth described in this paper.

I would like to thank Dr L. F. Hewitt for his interest in this work and I am grateful to Mr M. Roberts for skilled technical assistance.

REFERENCES

AINSWORTH, G. C., BROWN, A. M. & BROWNLEE, G. (1947). Aerosporin, an antibiotic produced by *Bacillus aerosporin*, Greer. *Nature, Lond.* **160**, 263.

ANDERSEN, E. K. (1952). Serological studies on *H. pertussis, H. para pertussis* and *H. bronchisepticus*; preliminary report. *Acta path. microbiol. scand.* **30**, 54.

BROWN, A. M. (1958). Intracerebral infection of mice with *Haemophilus pertussis* and passive protection by hyperimmune rabbit sera. *J. gen. Microbiol.* **18**, 48.

COHEN, S. M. & WHEELER, M. W. (1946). Pertussis vaccines produced with phase I cultures grown on fluid medium. *Amer. J. publ. Hlth*, **36**, 371.

JEBB, W. H. & TOMLINSON, A. H. (1955). The nutritional requirements of *Haemophilus pertussis*. *J. gen. Microbiol.* **13**, 1.

KENDRICK, P. L., ELDERING, G., DIXON, M. K. & MISNER, J. (1947). Mouse protection tests in the study of pertussis vaccine. A comparative series using the intracerebral route for challenge. *Amer. J. publ. Hlth*, **37**, 803.

LACEY, B. W. (1951). Antigenic modulation of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, xxi.

MAZLOUM, H. A. & ROWLEY, D. (1955). The growth requirements of *Haemophilus pertussis* on solid media. *J. Path. Bact.* **70**, 439.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of blood. *J. Hyg., Camb.* **38**, 732.

ROWATT, E. (1957). The growth of *Bordetella pertussis*: a review. *J. gen. Microbiol.* **17**, 297.

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Roberts, C. F. (1959). *J. gen. Microbiol.* **20**, 540–548

A Replica Plating Technique for the Isolation of Nutritionally Exacting Mutants of a Filamentous Fungus (*Aspergillus nidulans*)

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SUMMARY: This paper describes a replica plating technique for a filamentous fungus and shows its effectiveness in the isolation of nutritionally-exacting mutants, including mutants unable to grow with certain sugars as sole carbon source.

The two main applications of the replica plating technique are in the classification of colonies differing from each other in a number of known nutritional requirements, and in the isolation of new mutant strains. The first application is mainly in experiments on gene recombination where nutritional requirements are used as genetic characters. Organisms derived by recombination are plated so that each can give rise to a colony, and then each of the colonies is classified according to its combination of nutritional characters. It is not usually necessary to test more than a few hundred colonies for their response to a small number of nutrients. A convenient method for doing this (Mr E. C. Forbes, personal communication) is to prepare initial plates with a number of the colonies, perhaps 30, set in a standard pattern on each plate. These colonies are then replicated on to a series of test media by means of a multi-point inoculator, the points of which are also arranged in the standard pattern. The colonies are classified by their failure to grow on test media lacking particular nutrients. Such an application is now well known and has been used routinely in this laboratory for several years.

In the second application of replica plating, i.e. the isolation of mutants, the preparation of initial plates with an ordered pattern of colonies is impracticable because it is necessary to test many thousands of colonies, since mutants occur very infrequently. Lederberg & Lederberg (1952) resolved this difficulty by using a velveteen pad to replicate rapidly from initial plates bearing large numbers of randomly distributed colonies. Their technique and its variations have found wide usage in microbiology, and have been successfully applied to bacteria (Lederberg & Lederberg, 1952), to actinomycetes (Braendle & Szybalski, 1957) and to unicellular algae (Eversole, 1956).

Lederberg's replica plating technique has not been previously described in a form that can be applied readily to a filamentous fungus. Among the filamentous fungi *Aspergillus nidulans* has been extensively subjected to detailed genetic investigation (Pontecorvo *et al.* 1953; Käfer, 1958; Pontecorvo & Käfer, 1958) and it was desirable to have a straightforward replica plating technique for it. This paper describes the technique developed and its use in isolating nutritionally-exacting mutants of two types. One type are the usual auxotrophic mutants which can only grow when a particular nutrient (e.g. an amino acid, vitamin, etc.) is present in the medium. The other type are unable to utilize particular sugars as sole carbon sources for growth and are called 'sugar mutants'.

METHODS

Design of technique. The general method of replica plating was described by Lederberg & Lederberg (1952); it is useful to recall here the three essential stages. (1) Spreading a suspension of mainly separate organisms on a solid medium so that after incubation most of the colonies on the initial plate have grown from single organisms. (2) The use of a pad of sterile velveteen or similar rough material (e.g. filter-paper) to make replica inoculations from the initial plate on to a series of plates with different media ('test plates'). (3) Visual inspection of the series of replica plates to compare the responses of individual colonies to different media. There are a number of essential conditions for replica plating and factors which affect the efficiency of the technique. An obvious condition is that the organism should form discrete colonies on agar media. It is an advantage if these colonies are derived from uninucleate cells, and with fungi they should also sporulate reasonably well.

In the preparation of initial plates it is necessary to strike a balance between the number of colonies tested on each plate and the fact that with increased crowding of colonies the proportion of mutants that will not be detected because of mixed growth (syntrophy) also increases. Obviously organisms with nuclei which determine a nutritional requirement will only be detected when growing in a pure clone, and not when mixed with organisms which have unaltered nuclei and consequently are not exacting.

Choice of material for a replica pad is largely governed by the organism. In general a velveteen pad or a filter-paper disk are suitable for wettable spores or vegetative cells, especially bacteria, while dry spores are most effectively transferred on some type of metal prong.

In *Aspergillus nidulans* uninucleate haploid dry conidia are formed in profusion on solid media and suspensions of mainly separate conidia can easily be obtained. When conidia germinate close together on agar their hyphae tend to grow together and form mixed colonies. However, this does not occur between hyphae from conidia which are more than 0·5 cm. apart, and these conidia give rise to discrete colonies separated by zones of no growth. A Petri dish of 9·5 cm. internal diameter would accommodate *c.* 300 colonies if the conidia were at regular intervals of 0·5 cm., but in practice the upper limit, in order to avoid too many mixed colonies, is about 200 conidia on each initial plate.

Velveteen and filter-paper have proved only partly suitable for a replica pad (Dr E. Calef, personal communication). Moreover, with this type of pad only a single inoculum can be taken from the initial plate and this does not allow more than three replicas to be made. Eventually a 'replicator' similar to the multi-point inoculators used for *Aspergillus* to classify colonies by their nutritional requirements was chosen, but having many more points like the instrument for use with Streptomyces mentioned by Braendle & Szybalski (1957).

Construction of the replicator. One of the replicators is shown in Pl. 1, fig. 1. The base-plate is made from Perspex sheet in which holes that allow easy fitting of the steel pins (dressmaker's pins 2·5 cm. long) without letting their heads through are drilled in a regular pattern at 0·5 cm. intervals. It is important that the points of the pins should be in one plane, therefore the pins should be of equal lengths and the base-plate flat. The pins are held in position by Perspex cement, and a reference point to orientate the replica is provided by omitting a few of them from the pattern.

General method of application. To prepare the initial plates a dilute spore suspension is spread on solid medium to yield 100–200 colonies distributed evenly on each plate, and the plates incubated until good sporulation has taken place. The series of test plates are prepared with media chosen according to the purpose of replication (examples below) and dried to remove excess moisture. After sterilizing the replicator by dipping the pins in ethanol and igniting, the first initial plate is opened with the colonies upwards and the replicator lowered on to them. It is then withdrawn, laid on the bench with the pins uppermost and the first test plate inoculated by touching it against the points. The replicator can be recharged without further sterilization, and the sequence is repeated to inoculate each test plate of the series.

It is, of course, essential that all inocula are taken from exactly the same positions on the initial plate. This is readily achieved if the holes made in the initial plate when taking the first inoculum are used as guides for succeeding inoculations. Eight replica plates can be made from one initial plate without difficulty. With attention to normal aseptic routine contamination is negligible.

Strains and media. Strains of *Aspergillus nidulans* held in the Genetics Department of Glasgow University were used. Media and the general technique for handling *A. nidulans* given by Pontecorvo *et al.* (1953) were followed unless otherwise stated. Minimal medium (MM) was a solution of inorganic salts, including nitrate and sulphate as main nitrogen and sulphur sources, with 1% (w/v) glucose and 1·5% agar. Complete medium (CM) was a complex medium containing casein hydrolysate, yeast extract, various vitamins, a hydrolysate of yeast nucleic acid and the same concentrations of inorganic salts, glucose and agar as in MM.

RESULTS

A reconstruction experiment to illustrate the general application of the technique

Serial dilutions of a mixed suspension of conidia from three strains of *Aspergillus nidulans* with different conidial colours and nutritional requirements were spread on CM, incubated and plates with suitable numbers of colonies selected as initial plates. The replicator was used to inoculate a series of Petri dishes containing test media which in turn allowed only one of the three strains to grow, and finally on to CM on which they all grew. Plate 1, fig. 2, shows one of the sets of dishes; Table 1 gives details of strains, media and description of the photograph.

Table 1. *A reconstruction experiment to illustrate the general application of replica plating to* Aspergillus nidulans (*see also Pl.* 1, *fig.* 2)

Colonies of the three strains were grown together on the initial plate and replicas made on three test plates, each of which allowed only one of the three strains to grow, and finally on to CM on which they could all grow.

Strain*	Conidia	Nutritional requirement
*y*2; *pyro*4	Yellow	Pyridoxin
*bi*1; *w*3 *ad*3	White	Biotin, adenine
*paba*1; *ad*30	Green	*p*-Aminobenzoate, adenine

Plate		Medium	Incubation	Colonies growing
Initial	0	CM	4 days at 37°	All three strains
Replica	1	MM + pyridoxin	1 day at 37°	*y*2; *pyro*4
	2	MM + biotin + adenine	1 day at 37°	*bi*1; *w*3 *ad*3
	3	MM + adenine + *p*-aminobenzoate	1 day at 37°	*paba*1; *ad*30
	4	CM	2 days at 37°	All three strains

* Symbols of mutant alleles: (*a*) Requirements, *pyro*4 = pyridoxin; *bi*1 = biotin; *ad*3 and *ad*30 = adenine (different loci); *paba*1 = *p*-aminobenzoate. (*b*) Conidial colours, *y*2 = yellow; *w*3 = white; the wild type is green. (See Pontecorvo *et al.* 1953, for further details.)

In Pl. 1, fig. 2, orientation of the replicas is shown by three spots near the lower edge of the initial plate and three corresponding stabs or colonies on the other plates. Green, yellow and white colonies can be distinguished on plates 0 and 4 by their different shades. A number of mixed colonies on plate 4 have grown on more than one of the test plates, while plate 3 shows slight growth of strain *bi*1; *w*3 *ad*3 (point-like colonies) where close to colonies of other strains from which biotin was diffusing into the medium. It is clear from Pl. 1, fig. 2, that colonies with different nutritional requirements can be identified by their different growth responses when replicated on to the appropriate test media. Combination of nutritional requirements with different conidial colours allows the identification to be verified and provides a good illustration of the general application of replica plating to *Aspergillus nidulans.*

Reconstruction experiments with increasing numbers of colonies on the initial plates

Reconstruction experiments were done to investigate to what extent the efficiency of the technique decreases with increasing total number of colonies on the initial plates when, as in the actual isolation of mutants, the mutants sought represent a small proportion of all colonies. Mixed platings of two auxotrophic strains were used to simulate the situation when isolating nutritionally-exacting mutants, and to provide an estimate for the optimal density of colonies on initial plates when using the technique for this purpose. The two auxotrophic strains chosen had differently coloured conidia, to facilitate scoring. Suspensions of conidia of *Aspergillus nidulans* strains *y*2; *pyro*4 and *w*3 *ad*3 were mixed and plated on CM to give a series of initial plates with *c.* 10 colonies of *w*3 *ad*3 and 50–500 colonies of *y*2; *pyro*4 on each plate. (See Table 1 for explanation of symbols.) Replication was performed to see how many of the initial *w*3 *ad*3 colonies could be identified on the replica plates by their failure to grow on an adenine-deficient test medium. Replicas were therefore made from each initial plate on to MM + pyridoxin (no adenine) and on to CM (containing adenine). The test plates were compared after incubation for 24 hr. at 37° and the positions of colonies growing on CM but not on MM + pyridoxin marked. Incubation was continued and the scoring finally checked by inspection of conidial colours on the CM plates. A high proportion of colonies marked after 24 hr. as possible colonies of strain *w*3 *ad*3 proved to have been scored correctly.

Table 2. *Reconstruction experiments to show the loss of efficiency of the replica plating technique as the number of colonies on the initial plates increases*

Initial plates were prepared with about 10 colonies of *Aspergillus nidulans*, strain *w*3 *ad*3 and from 50 to 500 colonies of strain *y*2; *pyro*4. Replicas were made on to MM + pyridoxin and on to CM and compared after incubation for 24 hr. to identify colonies of strain *w*3 *ad*3.

Initial plates				Replica plates
		Total no. of colonies		
No. of plates	Mean no. of colonies/ plate	*y*2; *pyro*4	*w*3 *ad*3	No. of colonies of *w*3 *ad*3 identified on the replica plates
4	45	139	41	39 (95 %)
4	79	290	27	21 (78 %)
8	110	798	78	67 (86 %)
4	162	612	37	25 (68 %)
2	205	396	14	6 (43 %)
3	305	870	44	18 (41 %)
2	*c.* 500	*c.* 1000	28	6 (21 %)

Further experiments of the same type were performed by replicating directly on to CM and scoring by conidial colour alone. Those colonies which showed only white conidia under the binocular microscope were counted as recoveries. When *w*3 *ad*3 colonies on the replica plates formed obvious groups each group was counted as one as it probably represents a single colony on the initial plate. The data are presented in Table 2. It is apparent that as the total number of colonies on the initial plates increased fewer *w*3 *ad*3 colonies were identified on the test plates. At a density of 100–200 colonies on each initial plate (the average proportion of the rare component was 0·06 for these plates) identification was of the order of 70 % of the *w*3 *ad*3 colonies on the initial plates. In the isolation of nutritional mutants described below, the mutagenic treatment used is known from previous work to yield a proportion of 0·01 auxotrophs among the colonies growing from spores which survive the treatment. Therefore, though the reconstruction experiment does not exactly parallel the conditions used when isolating auxotrophs after mutagenic treatment, the figures indicate that about 70 % of nutritional mutants present on initial plates may be isolated by replica plating.

Isolation of auxotrophic mutants by replica plating

Screening experiments were done primarily to isolate sugar mutants, but auxotrophic mutants were isolated at the same time to provide some idea of the efficiency of the replica plating technique and to compare the frequencies of auxotrophic and sugar mutants.

Suspensions in saline of mainly separate conidia of *Aspergillus nidulans*, strains *y*2; *pyro*4 or *bi*1; *w*3, were irradiated under a u.v. lamp until the viable count was only 5% of the untreated controls, then diluted and plated on CM to yield 100–200 colonies/plate. After incubation for 3 days good sporulation had occurred and replicas were made on to MM + pyridoxin (or MM + biotin) and on to CM. Plates were compared after incubation for 24 hr. and isolates of colonies which failed to grow on MM + pyridoxin (or biotin) but which grew on CM were made from the CM plates. These isolates were retested and those which failed to grow at all on MM + pyridoxin (or + biotin) were purified by single colony isolation and classified for nutritional requirement by routine methods (Pontecorvo *et al.* 1953).

Table 3. *Auxotrophic mutants of* Aspergillus nidulans *isolated by replica plating, grouped into nutritional classes and compared with those obtained by other workers by total isolation*

Class of nutrient requirement	Replica plating		Total isolation*	
Nitrite or ammonia†	3	5%	17	18%
Thiosulphate or sulphite†	1	2%	7	8%
Amino acids	11	18%	12	13%
Components of yeast nucleic acid	6	10%	11	12%
Vitamins	17	31%	22	23%
Not tested	20	34%	24	26%
	58		93	
	6422 colonies on initial plates		5408 colonies tested	

* Data from Pontecorvo *et al.* (1953). The figures are not strictly comparable as different starting strains were used and they include strains obtained after irradiation with X-ray, and with u.v. radiation at survival rates other than 5%.

† The discrepancy between the two methods here is probably due to the 20 partial mutants obtained by replica plating which were not classified. Mutants unable to utilize nitrate or sulphate characteristically give partial growth on MM.

In this experiment 110 isolates were made from 49 initial plates bearing a total of 6422 colonies (4033 of strain *y*2; *pyro*4 and 2389 of strain *bi*1; *w*3). After retesting the yield was 58 mutants (0·9%) with a new nutritional requirement (36 from strain *y*2; *pyro*4 and 22 from strain *bi*1; *w*3). Twenty of these mutants were not tested further because the additional requirements for growth were only partial. The absolute requirements of the remaining 38 strains (0·6%) are given in the second column of Table 3, grouped by nutritional classes.

In a second experiment with strain *bi*1; *w*3 only auxotrophic mutants with a total nutritional requirement were isolated and the yield was therefore diminished, namely, 36 auxotrophs from *c.* 7400 colonies tested (0·5%). These auxotrophs have not been tested for their additional nutrient requirements.

The isolation of sugar mutants by replica plating

It was thought worth examining the possibility that strains of *Aspergillus nidulans* might occur which are unable to use certain sugars as sole carbon source. The screening experiments were carried out in conjunction with the isolation of auxotrophic mutants described above. Colonies on the initial plates were replicated on to series of seven test plates; one was minimal medium + 1% (w/v) glucose and the growth factors required by parent strain (the control), five were similar media but with glucose replaced by 1% galactose, lactose, maltose, sorbitol or sucrose, respectively; a final plate was complete medium. The auxotrophic mutants were detected by failure to grow on the control plate though growing on CM. The sugar mutants were detected by failure to grow on one or more of the five plates with the unusual sugars, though growing on the control plate (with glucose), the remaining sugars, and on CM. Strains presumed to be sugar mutants were isolated from the control plates, retested and purified by single colony isolation. The pure strains were finally tested by plating suspensions of separate conidia on control plates with glucose, and on media with the glucose replaced by the given sugar as commercially supplied or by this sugar twice recrystallized. Only those strains were retained which gave parent growth with glucose as carbon source but little or no growth with the sugar (as received or recrystallized) when colonies arose from single conidia.

Table 4. *Sugar mutants in* Aspergillus nidulans *isolated by replica plating*

The sugar mutants were isolated by replica plating and were detected by their failure to grow on a given sugar as sole carbon source.

Carbon source	No. of mutants	Minimum no. of loci
Sucrose	0	—
Maltose	2	1
Lactose	9	1
Galactose	8	1
Sorbitol	7	2
Fructose*	3	1
	29	6

* Mutants first isolated because of their failure to grow on sucrose or sorbitol.

In the experiments with *Aspergillus nidulans* strains *y*2; *pyro*4 and *bi*1; *w*3 9 sugar mutants (0·14%) were isolated from 6422 colonies tested by replica plating. In the second experiment with strain *bi*1; *w*3 20 sugar mutants (0·27%) were isolated from *c.* 7400 colonies. The distribution of these mutants is shown in Table 4, together with the number of loci that they are so far known to involve. The frequency of sugar mutants of types detectable by the tests in the two screening experiments was thus 29/13,800 (0·2%). In the same experiments the frequency of auxotrophic mutants with a clear-cut nutritional requirement was 74/13,800 (0·5 %). One or more mutants have been obtained for each of the sugars tested, except sucrose.

DISCUSSION

This paper describes the application of a replica plating technique to a filamentous fungus for the isolation of nutritionally-exacting mutants. The efficiency of the technique in recovering auxotrophic mutants of *Aspergillus nidulans* is about the same as that of the much more laborious method of total isolation (Pontecorvo *et al.* 1953). There should not be any difficulty in adapting the technique for work with other filamentous fungi. The main advantage of replica plating in the isolation of mutants is the saving of time and labour which enables a far larger population to be tested in a given time than could be done by the total isolation method. However, replica plating cannot be readily employed for quantitative studies and when an estimate of the numbers of a rare component in a population is required the total isolation method is preferable because every colony obtained after plating is known to have been tested once. In both replica plating and total isolation a fraction of the rare component may not be detected because of the growth of mixed colonies.

The replica plating procedure adopted for *Aspergillus nidulans* yielded auxotrophic mutants at the rate of 0·5–0·9 % of the colonies growing from conidia after 95 % killing by u.v. radiation; yields of the same order are obtained by total isolation after similar u.v. treatment. Reconstruction experiments to test the effectiveness of the procedure indicate a recovery of *c.* 70 % of colonies of the rarer type used on the initial plates. The procedure appears, therefore, to isolate almost as high a proportion of auxotrophic mutants as would the total isolation method under the same conditions. The distribution of mutants among the major nutritional classes does not show any obvious differences between the two methods, though the numbers investigated were small.

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REFERENCES

BRAENDLE, D. H. & SZYBALSKI, W. (1957). Genetic interaction among streptomycetes: heterokaryosis and synkariosis. *Proc. nat. Acad. Sci., Wash.* **43**, 947.

EVERSOLE, R. A. (1956). Biochemical mutants of *Chlamydomonas reinhardi*. *Amer. J. Bot.* **43**, 404.

KÄFER, E. (1958). An 8-chromosome map of *Aspergillus nidulans*. *Advanc. Genet.* **9**, 105.

LEDERBERG, J. & LEDERBERG, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.

Pontecorvo, G. & Käfer, E. (1958). Genetic analysis based on mitotic recombination. *Advanc. Genet.* **9**, 71.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advan. Genet.* **5**, 141.

EXPLANATION OF PLATE

Fig. 1. The replicator.

Fig. 2. Illustration of the application of replica plating to *Aspergillus nidulans*. Three strains with different nutritional requirements and with green, yellow or white conidia were grown together on the initial plate (0). Replicas were made on test media which allowed the strains with yellow (1), with white (2) or with green conidia (3) to grow. The last plate (4) contained medium on which all three strains grew. Colonies with differently coloured conidia can be distinguished on plates 0 and 4. Plates 1–3 distinguished colonies by their nutritional requirements and the correlation of conidial colour with nutritional requirement can be seen. (Table 1 gives details of the strains and media used.)

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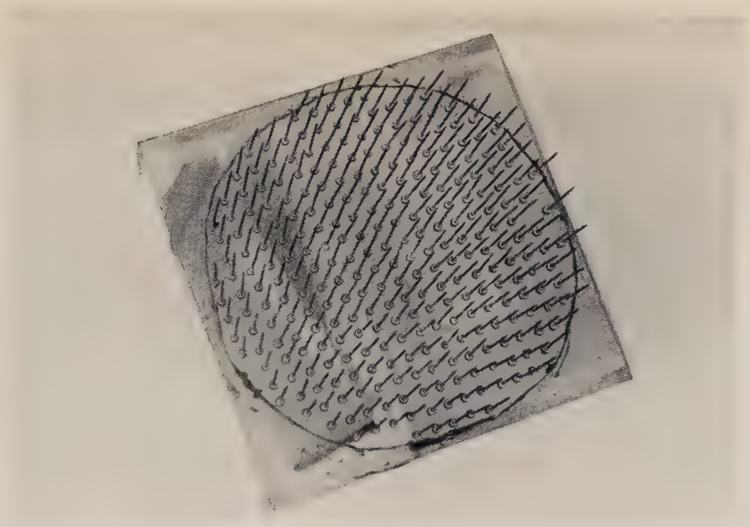

Fig. 1

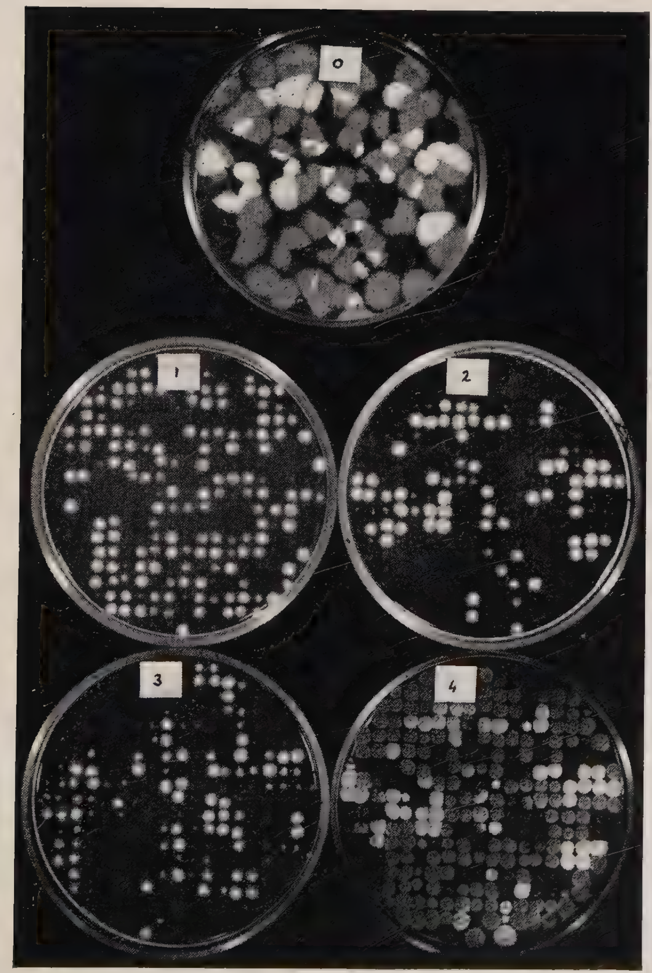

Fig. 2

C. F. ROBERTS—A REPLICA PLATING TECHNIQUE. PLATE 1

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Clarke, R. T. J. (1959). *J. gen. Microbiol.* **20**, 549–553

A Dextran-Fermenting Organism from the Rumen Closely Resembling *Lactobacillus bifidus*

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SUMMARY: An organism which ferments the dextran of *Streptococcus bovis* was isolated from the rumen of cows on a diet of fresh clover. The bacterium is a strictly anaerobic Gram-positive, non-sporing, branching rod that closely resembles *Lactobacillus bifidus*. It is of interest from the point of view of the metabolism of dextran in the rumen.

Hungate, Fletcher, Dougherty & Barrentine (1955) stated that bacterial slime production in the rumen 'may be associated in an important way with bloat in ruminants'. Bailey & Oxford (1958) showed that dextran is responsible for the greatly increased viscosity of sucrose-containing liquid cultures of rumen strains of *Streptococcus bovis*, and suggested that dextran slimes may be produced by *S. bovis* in the rumen of cows on a sucrose-containing ration such as spring grass or clover. As no dextran appears to accumulate in the rumen (R. W. Bailey, private communication) it is possible that it is broken down by microbial action. The isolations reported in this paper were made while searching for rumen organisms capable of attacking *S. bovis* dextran.

METHODS

Isolation of organism. Rumen contents were removed from fistulated cows and strained through gauze. Serial dilutions of the rumen liquor were made in dextran medium containing (%, w/v): Bacto-Tryptose, 1·0; Bacto-Yeast extract, 0·03; *Streptococcus bovis* dextran (Bailey & Oxford, 1958), 0·15; Davis N.Z. agar, 1·1. The medium was kept at 45° in 1 oz. bottles. Rumen liquor (1 ml.) was added to the first bottle, thoroughly mixed and 1 ml. transferred aseptically to the second bottle and so on for nine dilutions. The contents of each bottle were transferred aseptically to sterile tubes closed at one end with a rubber stopper. The open end was sealed with a 1:1 (v/v) mixture of paraffin wax and petroleum jelly, and the tubes incubated at 37° for 72 hr. The number of colonies present in the lowest dilution was noted. The agar was expelled and colonies picked out for plating on to dextran medium. The plates were incubated in a McIntosh & Fildes jar in an atmosphere of 95% (v/v) H_2 + 5% (v/v) CO_2. Subcultivation was continued until the cultures were considered to be pure.

Faeces were inoculated into the tomato juice broth of Rogosa *et al.* (1953) and incubated in an atmosphere of 95% (v/v) H_2 + 5% (v/v) CO_2 at 37° for 48 hr. Plates containing tomato juice broth solidified with 1·1% (w/v) agar were inoculated and incubated for 48 hr.

Cultures were maintained by frequent transfer in tomato juice medium with occasional passage through media containing dextran.

Identification of organism. The methods followed those of Rogosa *et al.* (1953). Fermentation tests were carried out in duplicate with washed suspensions from 48 hr. tomato juice cultures as inocula. Nitrate reduction was tested in ox heart infusion broth containing (%, w/v): KNO_3, 0·1; Bacto-Yeast extract, 0·3 (Costilow & Humphreys, 1955).

Lactic acid was determined by the method of Barker & Summerson (1941) and, together with the lower fatty acids, by partition chromatography on Celite with chloroform/butanol solvents (Swim & Krampitz, 1954).

RESULTS

Characters of organism isolated. Two strains were isolated from the rumen; strain D13/293 from cow no. 293 on a diet of fresh red clover, and strain D15/1 from cow no. 1 on a diet of fresh clover and clover hay. Strains D13/293 and D15/1 were present in the rumen at a concentration of 2×10^7 and 7×10^4 organisms/ml. rumen contents, respectively.

In liquid culture the organisms were slender, sometimes pleomorphic rods, 0·4–0·8 $\mu \times 2$–8 μ, occurring singly and in clumps; chain formation was not evident; Gram positive, but tending to stain irregularly in old cultures; non-motile. On solid media there was a large number of irregular, club, branched and Y-forms. Colonies on tomato juice agar and dextran agar plates were 0·5–2 mm. diameter, circular, convex, entire, smooth, white and opaque. Liquid cultures were turbid within 1–2 days, clearing with a flocculent sediment. Strictly anaerobic conditions were essential for growth; growth was better under 95% (v/v) H_2+5% (v/v) CO_2 than under 100% H_2. Over a period of 6 months no increase in tolerance to oxygen or change from the typical ‘bifid’ form was noticed.

The organisms were catalase negative, reduced nitrate to nitrite, did not liquefy gelatin and did not coagulate milk. The lactic acid formed from glucose was optically inactive. A 40–50% yield of volatile acid was produced from glucose in 72 hr. Gas production was not observed in the stab cultures of Rogosa *et al.* (1953).

The following carbohydrates were fermented in 48 hr. with the production of an acid pH value (numerical value in brackets): glucose (4·9), lactose (5·1), sucrose (5·1), fructose (4·9), maltose (5·3), xylose (5·6), arabinose (5·3), rhamnose (5·5), galactose (5·7), raffinose (5·3), cellobiose (5·2), starch (5·6), *Streptococcus bovis* dextran (5·2).

The following compounds were not fermented: inositol, mannose, mannitol, dulcitol, glycerol.

Growth of the organisms on dextran. The organisms grew profusely on a medium containing dextran as carbon source, producing lactic, formic and acetic acids. The enzyme responsible for the breakdown of the dextran was extracellular. It was obtained in a cell-free state and shown by paper chromatography to break the dextran molecule, characteristically, into tri-, tetra-, penta- and higher saccharides, a property useful for the identification of dextran. The properties of the enzyme are described elsewhere (Bailey & Clarke, 1959).

Comparison with strains of Lactobacillus bifidus. As the organisms appeared similar to *L. bifidus*, four strains of *L. bifidus* were obtained for comparison; strains D17/H, D18/H and D21/H from the faeces of nursing infants (a traditional source of *L. bifidus*) and strain D22/T from the faeces of a turkey.

All four strains of *Lactobacillus bifidus* were identical in morphology with the rumen organisms, exhibiting irregular, club, branched and Y-forms. All were obligate anaerobes and required CO_2 for growth. As with the two rumen organisms, no increased tolerance to oxygen or change to unbranched forms took place with continued sub-culture.

Strain D17/H was also similar in other respects to the rumen strains; fermentation reactions were the same, and the organism utilized dextran and reduced nitrates to nitrites. Strains D18/H, D21/H (human) and D22/T (turkey) did not attack dextran or reduce nitrates and did not ferment xylose, arabinose, rhamnose, cellobiose and starch, all of which were fermented by the two rumen organisms and by *Lactobacillus bifidus*, D17/H.

A strain labelled *Lactobacillus bifidus* (NCIB 2797) was also obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex. This organism was an unbranched micro-aerophilic rod resembling *L. brevis*. Glucose, maltose, fructose, xylose, arabinose and galactose were fermented, the fermentations of the pentoses being particularly vigorous. The organism also reduced nitrates to nitrites.

DISCUSSION

Two types of *Lactobacillus bifidus* have been isolated in past years. Tissier (1900) first described the organism ('*Bacillus bifidus*') from the faeces of breast-fed infants. The organisms were Gram-positive straight or curved rods, some exhibiting a split or bifid structure. On repeated subculture the organism lost its bifid appearance and became an unbranched rod. Eggerth (1935) and Orla-Jensen, Orla-Jensen & Winther (1936) described a second type of *L. bifidus* (*Bacteroides bifidus*; *Bacterium bifidum*) from the stools of adults. This organism maintained its bifid structure on subculture. Weiss & Rettger (1934, 1938) proposed that the two types should be classified as *L. bifidus* type I, and *L. bifidus* type II. Type I, first isolated by Tissier, usually becomes unbranched and a facultative anaerobe, while Type II, first isolated from adult faeces by Eggerth & Orla-Jensen, remains bifid and anaerobic. Much of the confusion still remaining was removed by Norris, Flanders, Tomarelli & György (1950). These authors cultivated *L. bifidus* indefinitely in a bifid phase and discussed differences between the bifid organisms and unbranched bacilli which corresponded with *L. bifidus* Type II and *L. bifidus* Type I, as described by Weiss & Rettger.

The two rumen organisms and the four strains of *Lactobacillus bifidus* described here correspond morphologically to *L. bifidus* type II of Weiss &

Rettger (1934, 1938). It should be noted that the rumen organisms produce optically inactive lactic acid from glucose, while the Type II strains (Weiss & Rettger) of Orla Jensen produce optically active acid. The optical activity of the lactic acid produced by the faecal strains was not tested. In spite of their morphological similarity, the rumen organisms and the *L. bifidus* strains are divisible into two groups. The restricted fermentation ability of *L. bifidus* strains D18/H, D21/H and D22/T, and their inability to reduce nitrates and to attack dextran distinguishes them from *L. bifidus* D17/H and rumen organisms D13/293 and D15/1. The fermentation range of both groups is within that listed for *L. bifidus* (Tissier, 1900) in *Bergey's Manual* (1957).

It is generally accepted that members of the genus *Lactobacillus*, with the exception of strains of *L. plantarum*, are unable to reduce nitrates. Costilow & Humphreys (1955) found that 18 of 38 strains of *L. plantarum* tested were able to reduce nitrates, and that nitrate reduction by some strains was enhanced by a decreased oxygen tension and by the addition of yeast extract to the medium. It is therefore not surprising that some strains of *L. bifidus* should reduce nitrates when grown anaerobically in the presence of yeast extract.

The only previous isolations of rumen organisms resembling *Lactobacillus bifidus* were made by Wasserman, Seeley & Loosli (1953). These authors isolated organisms, which they suggested were variants of *L. bifidus*, from dilutions of rumen contents higher than 10^{-6}. The organisms were catalase negative and homofermentative, producing optically inactive lactic acid from glucose. It was suggested that these variants of *L. bifidus* 'may be important converters of non-protein nitrogen to protein nitrogen in the rumen'. No study was made of their action on dextran.

This is the first occasion on which a member of the genus *Lactobacillus* has been shown to ferment dextran, and the second occasion on which extracellular dextranases have been reported in bacteria. Hehre & Sery (1952) demonstrated the formation of dextranases in members of the genus *Bacteroides*. The action of the enzyme preparations from these organisms produced glucose from dextran. Tsuchiya, Jeanes, Bricker & Wilham (1952) tested 200 species of bacteria and yeasts for dextranase activity with negative results. Unfortunately these authors did not list the species tested. Bailey & Clarke (1959) could not find any dextranase activity in single strains of four species of *Lactobacillus* other than *L. bifidus*.

The presence of dextran-attacking organisms in the rumen is to be expected when one considers the possible *in vivo* production of dextran by *Streptococcus bovis*. Dextran-attacking organisms could not be demonstrated in rumen contents during the winter when the cows were on a low sucrose diet. From these results it appears that *L. bifidus* is an important member of the rumen microflora only under certain conditions.

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REFERENCES

Bailey, R. W. & Clarke, R. T. J. (1959). A bacterial dextranase. *Biochem. J.* (in the Press).

Bailey, R. W. & Oxford, A. E. (1958). A quantitative study of the production of dextran from sucrose by rumen strains of *Streptococcus bovis*. *J. gen. Microbiol.* **19**, 130.

Barker, S. B. & Summerson, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535.

Bergey's Manual of Determinative Bacteriology (1957). 7th ed. Ed. by Breed, R. S., Murray, E. G. D. & Smith, N. R. Baltimore, Md. U.S.A.: Williams and Wilkins.

Costilow, R. N. & Humphreys, T. W. (1955). Nitrate reduction by certain strains of *Lactobacillus plantarum*. *Science*, **121**, 168.

Eggerth, A. H. (1935). The Gram-positive non-spore-bearing anaerobic bacilli of human faeces. *J. Bact.* **30**, 277.

Hehre, E. J. & Sery, T. W. (1952). Dextran-splitting anaerobic bacteria from the human intestine. *J. Bact.* **63**, 424.

Hungate, R. E., Fletcher, D. W., Dougherty, R. W. & Barrentine, B. F. (1955). Microbial activity in the bovine rumen: its measurement and relation to bloat. *Appl. Microbiol.* **3**, 161.

Norris, R. F., Flanders, F., Tomarelli, R. M. & György, P. (1950). The isolation and cultivation of *Lactobacillus bifidus*: a comparison of branched and unbranched strains. *J. Bact.* **60**, 681.

Orla-Jensen, S., Orla-Jensen, A. D. & Winther, O. (1936). *Bacterium bifidum* and *Thermobacterium intestinale*. *Zbl. Bakt.* (Abt. II), **93**, 321.

Rogosa, M., Wiseman, R. F., Mitchell, J. A., Disraely, M. N. & Beaman, A. J. (1953). Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius*, nov spec & *Lactobacillus cellobiosus* nov spec. *J. Bact.* **65**, 681.

Swim, H. E. & Krampitz, L. O. (1954). Acetic acid oxidation by *Escherichia coli*: evidence for the occurrence of a tricarboxylic acid cycle. *J. Bact.* **67**, 419.

Tissier, H. (1900). *Recherches sur la flore intestinale des nourrissons.* Thèse, Faculté de Medicine de Paris, Paris. Cited: Norris *et al.* (1950).

Tsuchiya, H. M., Jeanes, A., Bricker, H. M. & Wilham, C. A. (1952). Dextran-degrading enzymes from moulds. *J. Bact.* **64**, 513.

Wasserman, R. H., Seeley, H. W. & Loosli, J. K. (1953). The physiology and nutrition of a rumen Lactobacillus. *J. Anim. Sci.* **12**, 935.

Weiss, J. E. & Rettger, L. F. (1934). *Lactobacillus bifidus*. *J. Bact.* **28**, 501.

Weiss, J. E. & Rettger, L. F. (1938). Taxonomic relationships of *Lactobacillus bifidus* (*B. bifidus* Tissier) and *Bacteroides bifidus*. *J. infect. Dis.* **62**, 115.

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Wright, D. E. (1959). *J. gen. Microbiol.* **20**, 554–565

The Formation of Keto and Amino Acids by *Mycobacterium butyricum* Growing in a Chemically Defined Medium

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SUMMARY: α-Oxoglutaric, pyruvic and amino acids accumulated extracellularly when *Mycobacterium butyricum* was grown in a chemically defined medium containing glycerol as carbon source and ammonium sulphate as nitrogen source. The excretion of keto acids was diminished and that of amino acids was increased by raising the concentration of ammonium sulphate relative to that of glycerol. This change is consistent with the operation of a citric acid cycle which is performing synthetic functions. The labelling of the somatic amino acids formed in the presence of glycerol and either (^{14}C) bicarbonate or (2-^{14}C) acetate is also consistent with the occurrence of a citric acid cycle.

Numerous investigations of the nutritional requirements of the mycobacteria have shown that the ability to use glycerol and ammonium ions as major nutrients is common to most members of the genus (see Drea & Andrejew, 1953); *Mycobacterium butyricum* is no exception in this respect. Provided it is supplied with a small amount of thiamine, it grows well on a chemically defined medium in which glycerol and ammonium ions are the sole significant sources of carbon and nitrogen (Hunter, 1953). The exceptional feature of the growth of *M. butyricum* on this and on other media is the excretion of α-oxoglutaric acid and small amounts of pyruvic and succinic acids (Hunter, 1953). By using ^{14}C tracer methods, Edson, Hunter, Kulka & Wright (1959) found strong evidence to support the view that the α-oxoglutaric acid excreted by *M. butyricum* is derived from glycerol by way of the citric acid cycle and that the cycle plays an important part in both assimilation and dissimilation of glycerol. In particular, the labelling of the non-lipid carbon, including the protein-carbon, of the cells suggested that much of this carbon had passed through the cycle. The object of the present work was to study conditions under which keto acids are excreted and to investigate their relation to the synthesis of amino acids.

METHODS

Organism. Mycobacterium butyricum (no. 337) was obtained from the National Collection of Type Cultures, then at the Lister Institute, London. Flasks of medium were inoculated from culture maintained on the chemically defined medium by serial transfer every fourth day.

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Chemically defined medium. The chemically defined medium contained (g.): 1·0, $(NH_4)_2SO_4$; 0·2, NaCl; 0·04, $MgSO_47H_2O$; 10·0, KH_2PO_4; 0·01, thiamine hydrochloride; 30 glycerol (analytical reagent); distilled water to 1 l. The pH value was adjusted to 7·5 with 3N-NaOH and the medium sterilized by autoclaving at 121° for 15 min.

Growth experiments. Conical flasks (500 ml.), each containing 100 ml. of medium, were inoculated with 2 mg. wet wt. of organism and incubated at 38°. The bacterial growth was estimated by filtering-off the pellicle on Whatman no. 1 paper, washing three times with distilled water and drying at 105°. For the purposes of this study the small loss of organism resulting from using this method was not significant. The culture filtrate and washings were combined and centrifuged to remove any remaining organisms.

pH *values.* All pH estimations were made colorimetrically with a Lovibond comparator box.

Glycerol was estimated by the method of Lambert & Neish (1950).

α-Oxoglutaric and pyruvic acids were measured by the method of El Hawary & Thompson (1953); total keto acids were estimated according to Friedemann & Haugen (1943).

Total nitrogen. Bacteria and samples of culture filtrates were digested (Hiller, Plazin & Van Slyke, 1948) and the digest transferred to a Markham apparatus along with two drops of tributylcitrate and 1 ml. 10N-NaOH. The ammonia was distilled into 10 ml. of 2% borate buffer and titrated with standard HCl.

Free ammonia. Samples of the culture filtrates were distilled directly and titrated as above.

Amide nitrogen. Samples of culture filtrates were heated in a boiling water bath with HCl (final concentration of 2N-HCl) for 1 hr., the solutions distilled after adding alkali and the distillate titrated. The difference in titre between this value and the free ammonia value represented amide nitrogen.

Amino nitrogen. Portions of the culture filtrates were treated with HNO_2 in a micro-Van Slyke apparatus as described by Harrow *et al.* (1944).

Steam volatile acids. Portions of the culture filtrates were acidified and steam distilled. Carbon dioxide was removed by refluxing the distillate which on cooling was titrated with standard alkali. Acetic acid was the only acid detected in these samples by paper chromatography (Reid & Lederer, 1952).

Chemicals. Most of the inorganic and organic chemicals were analytical reagents. Sodium (^{14}C) bicarbonate and sodium (2-^{14}C) acetate were obtained from the Radiochemical Centre, Amersham, England.

Chromatography of amino acids

Preparation of samples. (1) *Cell hydrolysates.* The bacteria were defatted (Edson *et al.* 1959) and samples (20 mg.) of the residue hydrolysed in sealed tubes with 5 ml. of 6N-HCl at 100° for 8 hr. The HCl was removed by repeated evaporation and the amino acids dissolved in 1 ml. of water. (2) *Cell extracts.* Samples (50 mg.) of the dried organisms were heated with 5 ml. of distilled water in a boiling water bath for 10 min. (Gale, 1947), the organisms centrifuged down and the supernatant fluid concentrated for chromatography. (3) *Amino acids in culture filtrates.* A portion (50 ml.) of culture filtrate was concentrated to 10 ml. in a rotary evaporator at 30° and an equal volume of cold ethanol added. After standing at 4° for 1 hr., the solution was centrifuged, the supernatant fluid concentrated to 5 ml. and the process of precipitation repeated. The supernatant fluid was concentrated to a thick syrup and dissolved in 5 ml. of ethanol water (1:1). This procedure removed sufficient inorganic and protein material to give satisfactory chromatograms. The presence of glycerol sometimes distorted the spots.

Paper chromatography. The amino acids were analysed by two-dimensional paper chromatography using *sec.*-butanol: formic acid: water (70:10:20) as solvent for the first dimension and phenol: water: concn. ammonia (sp.gr. 0·88; 80 g.:20 ml.:0·3 ml.) for the second dimension (Roberts *et al.* 1955). Ascending chromatography was used, the solvents reaching the top of the paper in about 16 hr. Hydrogen peroxide and ammonium molybdate were used to allow separation of methionine and valine (Dent, 1948). The chromatograms were sprayed with ninhydrin reagent. Radioautographs were prepared by placing paper chromatograms in contact with Ilford X-ray films and exposing for periods up to two months.

RESULTS

The growth of Mycobacterium butyricum *in the defined medium*

Little growth was observed until about the third day when the surface of the medium was covered by a thin pellicle which later became pigmented and thickened. Maximum growth was attained by the seventh to eighth day, after which the weight of organism decreased, indicating autolysis. Glycerol was consumed throughout the test period, the rate of utilization diminishing after 7 days (Fig. 1). Although growth was considerable by the fifth day, only small amounts of α-oxoglutaric and pyruvic acids had accumulated in the medium by this time. Maximum keto acid accumulation, due mainly to α-oxoglutaric acid, was found at 11 days. In 13 days the pH value of the medium fell from 7·4 to 5·2 in spite of heavy phosphate buffering (Fig. 2).

The influence of glycerol concentration. The carbon requirement of the organism was studied by varying the glycerol concentration in the range from 2 to 5% (w/v). There was no significant effect on the yield of organism or on keto acid accumulation when the initial concentration of ammonium sulphate was either 0·05 or 0·1 g./100 ml. (Table 1). In Expt. 3 (Table 1) it appears that nitrogen deficiency had restricted growth but not the excretion of keto acids. On the basis of these results a glycerol concentration of 3% (w/v) was chosen for further study of keto acid formation.

The influence of ammonium sulphate concentration. Table 2 shows that increasing the ammonium sulphate concentration above 0·1 g./100 ml. in the presence of 3 or 4% (w/v) glycerol lead to a greater consumption of glycerol, an increased yield of organism and a striking diminution in the excretion of keto acids (cf. Table 1). The amount of keto acid which accumulated was inversely proportional to the original nitrogen content of the medium but directly proportional to the glycerol: ammonia ratio. The observations on the medium which originally contained 2% (w/v) glycerol (Table 2) show the same trends but are not strictly comparable because the glycerol was exhausted or almost exhausted at the end of the experiments.

The final pH value of the medium bore no regular relation to its initial composition or to the accumulation of keto acids.

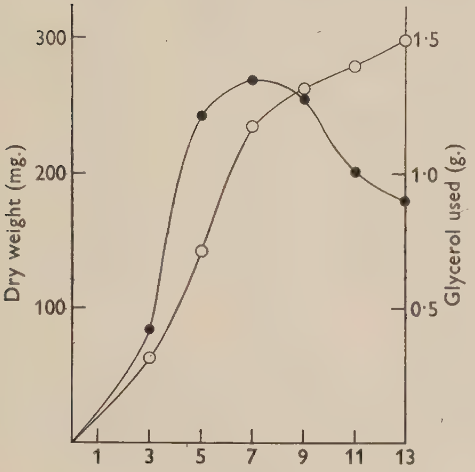


Fig. 1

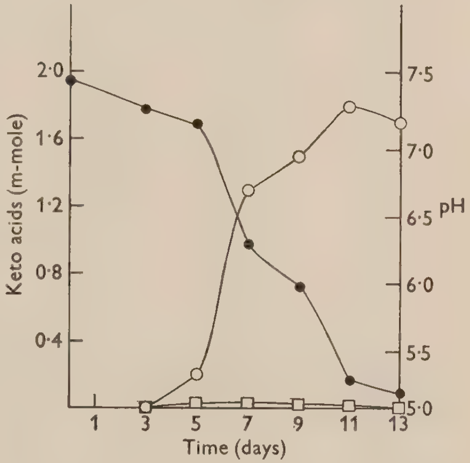


Fig. 2

Fig. 1. Growth and glycerol consumption of *Mycobacterium butyricum* in the defined medium. ●——●, dry weight of organism; ○——○, glycerol consumption.

Fig. 2. Keto acid accumulation and pH changes in the defined medium. ●——●, pH value; ○——○, α-oxoglutaric acid; □——□, pyruvic acid.

Table 1. *The effect of glycerol concentration on growth and keto acid formation by* Mycobacterium butyricum

The medium (see Methods) contained 0·1 g. ammonium sulphate/100 ml. in Expts. 1 and 2 and 0·05 g./100 ml. in Expt. 3. The inocula were taken from different mother cultures in each experiment.

Expt.	Time (days)	Glycerol (g./100 ml.)	Final pH value	Glycerol used (g.)	Bacterial growth (mg. dry wt.)	Total keto acid (m-mole)
1	9	3	6·3	1·1	242	1·3
		4	6·4	1·4	223	1·2
		5	6·3	1·7	238	1·3
2	10	2	6·4	1·1	212	1·5
		3	6·5	1·1	203	1·3
		4	6·6	1·1	202	1·1
3	10	2	6·7	0·8	140	1·1
		3	6·5	0·9	146	1·2
		4	6·6	1·0	141	1·3

Table 2. *The effect of varying the glycerol:nitrogen ratio*

100 ml. of medium. The ammonium sulphate was added aseptically to the sterile basal medium. Period of incubation: 10 days.

Glycerol (g./100 ml.)	Initial medium $(NH_4)_2SO_4$ (g./100 ml.)	Glycerol: ammonia-N (mole)	Final pH value	Glycerol used (g.)	Bacterial growth (mg. dry wt.)	Total keto acid (m-mole)
2	0·20	7·1	6·2	2·0	358	0·27
2	0·25	5·7	6·0	1·7	353	0·12
2	0·30	4·8	6·0	2·0	346	0·04
3	0·15	14·3	6·1	2·2	338	0·80
3	0·20	10·7	6·4	2·1	358	0·09
3	0·25	8·6	6·4	1·9	315	0·09
3	0·30	7·1	6·2	2·1	347	0·04
4	0·15	19·1	6·3	2·4	348	0·99
4	0·20	14·3	6·3	2·4	395	0·52
4	0·25	11·4	5·9	2·1	379	0·17
4	0·30	9·5	6·1	2·1	371	0·04

Nitrogen balance

Since α-oxoglutaric acid is formed from glycerol, its accumulation in a medium with a high initial glycerol:ammonia ratio and its absence from a medium with a lower ratio might be explained by the effect of available nitrogen on amino acid synthesis. This possibility was explored by constructing nitrogen balance sheets for media containing 3·0 g. glycerol/100 ml. and different amounts of ammonium sulphate. The ammonia added is expressed as μ-atom N/100 ml. of medium and to facilitate description the levels of nitrogen have been designated 1 to 6 in order of increasing quantity (Table 3). The recovery of nitrogen was satisfactory (98–102 %).

At nitrogen levels below 4 the ammonia was exhausted, growth was restricted and the nitrogen content of the organisms was low. At level 4 the organisms appeared to have attained maximum growth and nitrogen content. At level 1 the glycerol consumption was 0·9 g.; at all other levels it was 1·6 g.

Less than half the ammonia used at levels 4, 5 and 6 reappeared as cell-nitrogen. Above level 2, the residual medium contained amino nitrogen which increased with the amount of ammonia consumed. The small amount of protein released into the medium cannot account for this amino nitrogen. Amide nitrogen was not detected in any of the media. The nitrogen fraction designated 'other forms' includes protein (cf. Edson *et al.* 1959) and peptide nitrogen and unidentified nitrogen compounds.

Keto acids were present in all the residual media, the quantity being greatest where little or no ammonia remained (Table 3). The pH value was 6·8 at nitrogen level 1 and 6·5 at the other levels. The excretion of α-oxoglutaric acid was high at level 1 in spite of restricted growth, reached a maximum at level 2 and was completely absent at levels 5 and 6. Pyruvic acid accumulated to the greatest degree at level 4. The inverse relationship between the keto acid and amino nitrogen content of the various media could be explained if the keto acids were replaced by amino acids, especially glutamic acid, as the supply of nitrogen was increased. Such a phenomenon could be consistent with a relatively constant terminal pH value.

Table 3. *Nitrogen balance studies*

100 ml. of medium containing 3·0 g. glycerol. Period of incubation: 11 days.

Nitrogen level	Ammonia-N added (μ-atom)	Bacterial-N (μ-atom)	Ammonia-N (μ-atom)	Amino-N (μ-atom)	Other forms* of N (μ-atom)
1	362	307	0	0	55
2	722	607	0	0	115
3	1,448	992	0	93	363
4	2,896	1,372	86	558	880
5	5,772	1,363	2,700	672	1,037
6	11,584	1,336	8,440	736	1,072

Nitrogen level	α-Oxoglutaric acid (μmole)	Pyruvic acid (μmole)	Growth (mg. dry wt.)	Cell-N (% dry wt.)	N-recovery (%)
1	870	0	86	5·0	99
2	1,650	120	174	4·9	102
3	470	210	229	6·1	100
4	90	560	270	7·1	99
5	0	90	279	6·9	99
6	0	80	260	7·2	98

* Calculated by difference.

Note. In relating these results to those of Table 2 it may be noted that 0·2 g. of $(NH_4)_2SO_4$/100 ml. is equivalent to 3030 μ-atoms N/100 ml.

Synthesis of amino acids at different levels of nitrogen supply

Hydrolysates of organisms. The organism was grown for 11 days in media containing 3·0 g. glycerol/100 ml. and ammonium sulphate at the nitrogen levels 2, 3 and 5 of Table 3. The washed, defatted organisms (see Methods) were subjected to acid hydrolysis and the amino acids, derived chiefly from the somatic proteins, analysed by paper chromatography. The amino acids identified were aspartic acid, glutamic acid, diaminopimelic acid, serine, glycine, threonine, alanine, lysine, tyrosine, methionine, arginine, proline, valine, leucine + isoleucine and phenylalanine. There were no qualitative differences in the amino acid composition of organisms grown at the different nitrogen levels.

Extracts of organisms. The free amino acids extracted from washed organisms by hot water were also examined. Organisms grown for 11 days at low nitrogen levels (1 and 2 of Table 3) released traces of glutamic acid, glycine, alanine and unidentified material giving two ninhydrin-reacting spots. At higher nitrogen levels the quantities of these three amino acids were increased and additional spots due to lysine, aspartic acid, methionine, valine and arginine were observed.

Culture filtrates. No amino acids were detected by paper chromatography in culture filtrates at the three lower nitrogen levels of Table 3. At level 4 glutamic acid, lysine, valine and leucine+isoleucine were present in the medium; at the higher nitrogen levels glycine, alanine, methionine and arginine appeared in addition. In all the positive samples glutamic acid was present in considerably greater amounts than the other amino acids.

Effect of adding sodium acetate to the medium

Although washed suspensions of *Mycobacterium butyricum* oxidize acetate easily (Hunter, 1953), the organism does not grow to a significant extent in a medium in which acetate is the sole carbon source (Edson & Hunter, 1943). Nevertheless, when it is grown on glycerol it utilizes (^{14}C) acetate with ease (Edson *et al.* 1959).

A small amount (0·1–0·3 m-mole) of steam-volatile acid identified as acetic acid appeared in the medium when *Mycobacterium butyricum* grew on glycerol. When a correction is made for this endogenous acetate, the rate of disappearance of acetate was shown by an experiment in which 8-m-mole acetate were added to the glycerol-containing medium before inoculation. After incubation for 8 days 1·1 m-mole of added acetate remained in the medium and none was left on the 11th day.

Table 4 shows the effect of added acetate on growth and on keto acid excretion. The lower concentrations of acetate (2–6 m-mole/100 ml.) did not affect growth significantly but increased the excretion of pyruvic acid and diminished that of α-oxoglutaric acid. The higher concentrations of acetate (7–10 m-mole/100 ml.) enhanced growth and the yield of α-oxoglutaric acid. At 10 m-mole acetate/100 ml. the change in the keto acid excretion appears to have occurred at the expense of pyruvic acid.

Table 4. *The effect of adding acetate to the defined medium*

100 ml. of medium containing 3·0 g. glycerol and 0·1 g. $(NH_4)_2SO_4$. 2N-sodium acetate solution was added aseptically. Period of incubation: 14 days.

Acetate added (m-mole)	Final pH value	Bacterial growth (mg. dry wt.)	α-Oxoglutaric acid (m-mole)	Pyruvic acid (m-mole)
0	6·5	143	1·2	0·4
2	6·5	148	0·6	0·7
4	7·1	144	0·2	0·6
5	7·3	154	0·3	1·1
6	7·3	140	0·5	1·6
7	7·2	206	1·0	1·6
8	6·6	201	2·2	3·8
10	6·0	249	6·6	0·2

Synthesis of amino acids from (2-^{14}C) *acetate and* (^{14}C) *bicarbonate*

Mycobacterium butyricum was grown in 30 ml. defined medium containing 3·0 g. non-isotopic glycerol and 0·4 g. ammonium sulphate/100 ml. The culture flask (100 ml.) was attached to a pump by means of which a stream of CO_2-free air was drawn through the apparatus and respired CO_2 was trapped in a bubbler containing 3 N-NaOH solution. The flask was provided with a glass-stoppered side-arm through which sterile solutions of ^{14}C-containing substrates could be added to the medium.

Sodium (^{14}C) *bicarbonate.* A small volume (0·4 ml.) of 0·5 M sodium (^{14}C)-bicarbonate solution (sterilized by filtration) with a specific activity of $9{\cdot}23 \times 10^6$ counts/min./m-mole was added to the medium of the second, third, fourth and fifth days after inoculation. Aeration was intermittent in order that some $^{14}CO_2$ should remain in the flask during the greater part of the experiment.

The organisms were removed on the sixth day, dried at 105°, defatted and hydrolysed in acid (see Methods). The amino acids derived from the somatic proteins were separated by paper chromatography and radioautographs were prepared. The radioactive areas of the paper were located by reference to the radioautograph and the spots cut out. These were mounted on a holder which exposed 6·3 cm.^{2} of paper and the radioactivity from both sides of the paper disks were counted under a thin end-window Geiger-Müller tube. Sufficient counts were taken to give a standard error of counting of $\pm 3\%$. The mean counts for the two sides are recorded in Table 5, there being no significant differences between the two counts. Radioactivity was detected in the amino acids in the culture filtrates but the concentration of amino acids was too low to permit measurement of the radioactivity.

Table 5. *The isotope content of amino acids formed in the presence of* (^{14}C) *bicarbonate and* (2-^{14}C) *acetate*

The counts/minute have been corrected for background.

Amino acid	(2-^{14}C)Acetate (counts/min.)	(^{14}C)Bicarbonate (counts/min.)
Diaminopimelic acid	33	45
Aspartic acid	91	148
Glutamic acid	316	247
Serine	15	11
Glycine	16	41
Threonine	53	74
Alanine	62	0
Tyrosine	9	0
Lysine	51	47
Arginine	57	208
Proline	93	97
Leucine + isoleucine	116	41

Sodium (2-^{14}C) *acetate.* On the first and fourth days after inoculation 0·9 ml. (1·25 m-mole) of an autoclaved solution of (2-^{14}C) acetate (specific activity 6×10^5 counts/min./m-mole of (2-^{14}C) acetate) was added to the medium. The apparatus was flushed with CO_2-free air at least three times daily to remove as much respiratory CO_2 as possible and the bacteria collected on the sixth day. They were hydrolysed and radioautographs of the amino acids prepared as before. The counts of the paper chromatograms are given in Table 5.

The most heavily labelled of the amino acids formed by cells grown in the presence of (^{14}C) bicarbonate were glutamic acid, arginine, aspartic acid and proline. No radioactivity was detected in the alanine spot. The distribution of ^{14}C from acetate led to the heaviest labelling in glutamic acid; next in order were leucine+isoleucine, aspartic acid and proline. In this case alanine was labelled.

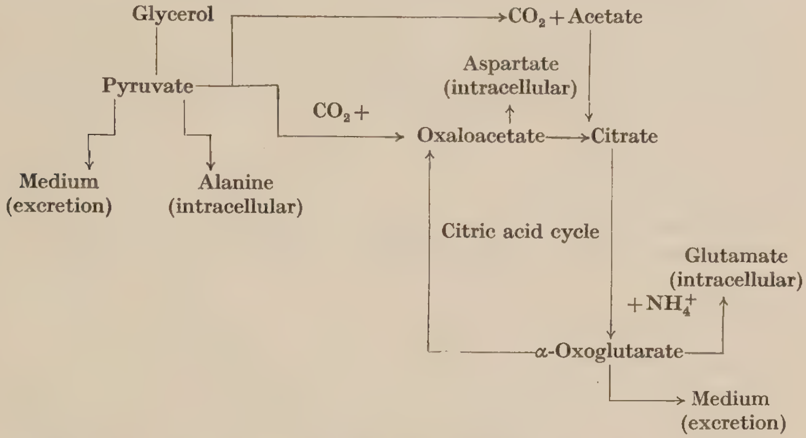
These experiments show that *Mycobacterium butyricum* utilizes carbon dioxide and the methyl group of acetate to form certain amino acids whose carbon skeletons are known to be derived from intermediates of the citric acid cycle in other organisms. The relatively high count of arginine formed in the presence of $^{14}CO_2$ indicates that carbon dioxide fixation takes place by well-established pathways. The labelling of glycine and serine has a particular interest. The isotope content of both was low when the ^{14}C was derived from (2-^{14}C) acetate, but when it was derived from $^{14}CO_2$ the glycine was more heavily labelled than the serine. The absence of isotopic equilibrium in this case could be explained if the glycine were formed directly from (1-^{14}C) glyoxylate. This is possible because *M. butyricum* contains isocitritase (Morrison & Wright, 1958) and $^{14}CO_2$ is fixed in the requisite carboxyl group of isocitrate (Edson *et al.* 1959).

DISCUSSION

Traces of keto acids have been found in defined media on which tubercle bacilli were grown (Boissevain, 1943; Yonemura & Iizuka, 1955*a*), the quantities being much smaller than those accumulated by *Mycobacterium butyricum*. On the other hand, *Pseudomonas fluorescens* excretes larger quantities of α-oxoglutaric acid than *M. butyricum* (Lockwood & Stodola, 1946) but under appropriate conditions its behaviour closely resembles that of *M. butyricum*. With *P. fluorescens* strain KB_1, Kogut & Podoski (1953) found no α-oxoglutaric acid in the medium unless the ratio of carbon (succinate) to nitrogen (ammonium ions) was made very large. Thus the excretion of keto acids is evoked in both organisms by restricting the available nitrogen supply relative to that of carbon (see Tables 2 and 3).

Free amino acids, especially glutamic acid, are released into the medium during the growth of tubercle bacilli on media which contain ammonium salts (Pauletta & Defranceschi, 1952; Yonemura & Iizuka, 1955*b*; Shoji, Yamagami & Mori, 1957). This appears to be due partly to breakdown of the organisms in the stationary phase and partly to amino acid excretion in earlier phases of culture. Dagley & Johnson (1956) have related the excretion of free amino acids to particular growth phases of *Escherichia coli* and other organisms. Although this has not been done with *Mycobacterium butyricum*, it seems unlikely that the release of amino acids is due solely to autolysis in experiments which last 11 days (cf. Fig. 1 and Table 3). Amino acids were not released in significant amounts at low levels of nitrogen nutrition; and when they were released at high levels of nitrogen nutrition there was a reciprocal relation with keto acid excretion. This relation could be explained if the citric acid cycle has a major role in the synthesis of amino acids. The distribution of ^{14}C amongst the somatic acids supports this view (Table 5). Aspartic and glutamic acids, which are known to be most intimately associated with the citric acid cycle, were heavily labelled and members of their postulated families (diaminopimelic acid-lysine-threonine-isoleucine and arginine-proline respectively) were also radioactive (cf. Roberts *et al.* 1957).

The relevant flow of carbon in the citric acid cycle during growth of *Mycobacterium butyricum* in the defined medium may be represented by the following scheme which neglects the oxidative function of the cycle.



So long as cellular constituents are synthesized at a rate which is at least equal to the rate of carbon influx from glycerol, keto acids should not be excreted into the medium. The ammonia content of the medium will be a limiting factor in protein synthesis. Hence keto acid accumulation will not be expected in the early stages of growth even in a nitrogen-poor medium. If the culture continues to metabolize glycerol after it has attained maximum growth, or if the rate of glycerol breakdown exceeds the capacity for cell component synthesis during growth, carbon could flow from the cycle to the exterior either as keto acid or as amino acid, depending on the concentration of ammonia present in the medium contemporaneously.

The experimental observations are consistent with this interpretation. The accumulation of α-oxoglutaric acid occurs late in the growth of *Mycobacterium butyricum* on a medium with a high glycerol:ammonia ratio and continues after maximum growth has been attained (Fig. 2); the addition of acetate to a glycerol-containing medium increases the outflow of carbon as keto acids (Table 4); and diminution of the glycerol:ammonia ratio in the medium suppresses the excretion of keto acids but leads to increased excretion of amino acids (Table 3) amongst which glutamic and aspartic acids are prominent. The excretion of pyruvic acid could be attributed to withdrawal from the pyruvate pool, especially in the presence of added acetate which might spare the direct oxidation of pyruvate; on the other hand, the excreted pyruvic acid might represent a withdrawal of oxaloacetate from the cycle, since oxaloacetate is readily broken down by a α-carboxylase known to be present in acid-fast bacteria (Morrison, 1956).

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REFERENCES

Boissevain, C. (1943). Survival of tubercle bacilli in solutions containing glycerol or its oxidation products. *Proc. Soc. exp. Biol., N.Y.* **54**, 342.

Dagley, S. & Johnson, A. R. (1956). Appearance of amino acids and peptides in culture filtrates of micro-organisms growing in mineral salt media. *Biochim. Biophys. Acta*, **21**, 270.

Dent, C. E. (1948). A study of the behaviour of some sixty amino-acids and other ninhydrin reacting substances on phenol-'collidine' filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.* **43**, 169.

Drea, W. F. & Andrejew, A. (1953). *The Metabolism of the Tubercle Bacillus.* Illinois: Charles C. Thomas.

Edson, N. L. & Hunter, G. J. E. (1943). Respiration and nutritional requirements of certain members of the genus *Mycobacterium. Biochem. J.* **37**, 563.

Edson, N. L., Hunter, G. J. E., Kulka, R. G. & Wright, D. E. (1959). The intermediary metabolism of glycerol in *Mycobacterium butyricum. Biochem. J.* (in the Press).

El Hawary, M. F. S. & Thompson, R. H. S. (1953). Separation and estimation of blood keto acids by paper chromatography. *Biochem. J.* **53**, 563.

Friedemann, T. E. & Haugen, G. E. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.

Gale, E. F. (1947). The assimilation of amino acids by bacteria. 1. The passage of certain amino-acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis. J. gen. Microbiol.* **1**, 53.

Harrow, B., Stone, G. C. H., Borek, E., Wagriech, H. & Mazur, A. (1944). *Laboratory Manual of Biochemistry.* U.S.A.: W. Saunders and Co.

Hiller, A., Plazin, J. & van Slyke, D. D. (1948). A study of conditions for Kjeldahl determination of nitrogen in proteins. Description of methods with mercury as catalyst, and titrimetric and gasometric measurements of the ammonia formed. *J. biol. Chem.* **176**, 1401.

Hunter, G. J. E. (1953). Acid formation in cultures of *Mycobacterium butyricum. J. Bact.* **66**, 676.

Kogut, M. & Podoski, E. P. (1953). Oxidative pathways in a fluorescent *Pseudomonas. Biochem. J.* **55**, 800.

Lambert, M. & Neish, A. C. (1950). Rapid method for estimation of glycerol in fermentation solutions. *Canad. J. Res.* B, **28**, 83.

Lockwood, L. B. & Stodola, F. H. (1946). Preliminary studies on the production of α-ketoglutaric acid by *Pseudomonas fluorescens. J. biol. Chem.* **164**, 81.

Morrison, N. E. (1956). Oxidation of malate by *Mycobacterium smegmatis. Proc. Univ. Otago Med. Sch.* **34**, 23.

Morrison, N. E. & Wright, D. E. (1958). Isocitratase activity in *Mycobacterium butyricum. Proc. Univ. Otago Med. Sch.* **36**, 1, 15.

Pauletta, G. & Defranceschi, A. (1952). Studies on the amino acid metabolism of *Mycobacterium tuberculosis.* I. Primary investigation by paper chromatography. *Biochim. Biophys. Acta*, **9**, 271.

Reid, R. L. & Lederer, M. (1952). Separation and estimation of saturated C_2–C_7 fatty acids by paper partition chromatography. *Biochem. J.* **50**, 60.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). Studies of biosynthesis in *Escherichia coli. Publ. Carneg. Instn*, no. 607.

SHOJI, K., YAMAGAMI, A. & MORI, T. (1957). Studies on the amino acid metabolism of *Mycobacterium tuberculosis*. I. Utilisation of some nitrogenous substances and accumulation of free glutamic acid in bacterial cells. *Med. J. Osaka Univ.* 7, 787.

YONEMURA, T. & IIZUKA, M. (1955*a*). Studies on the metabolism of amino acids in tubercle bacilli. I. The changes of free amino acids in liquid synthetic media following the growth of bacilli. *Jap. J. Tuberc.* 3, 6.

YONEMURA, T. & IIZUKA, M. (1955*b*). Studies on the metabolism of amino acids in tubercle bacilli. II. On the mechanism of the appearance of free amino acids in the medium. *Jap. J. Tuberc.* 3, 22.

(*Received* 24 *November* 1958)

Morris, J. G., Hughes, D. T. D. & Mulder, C. (1959). *J. gen. Microbiol.* **20**, 566–575

Observations on the Assay of Vitamin B_6 with *Saccharomyces carlsbergensis* 4228

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SUMMARY: The strain of *Saccharomyces carlsbergensis* 4228 studied differed from those previously used for the assay of vitamin B_6 in that it required this vitamin for rapid growth in the absence of added thiamine. The rate, rather than the ultimate extent, of growth of the organism is proportional to the concentration of vitamin B_6, and the size of the inoculum and its physiological age, the duration and temperature of incubation and the degree of aeration, must be strictly controlled. Non-phosphorylated forms of the vitamin only are utilized, and natural materials are acid-extracted before assay. The organism responds a little less well to pyridoxamine than it does to pyridoxal and pyridoxine. A linear response curve is obtained over a range of pyridoxine concentrations ($0{\cdot}2$ to 2×10^{-8}M) when incubation is in static culture at 37° (24 hr.) in a basal medium slightly modified from those previously used.

Although several accounts (Hopkins & Pennington, 1947; Snell, 1950; Fournier, Thomas & Seigel, 1951; Parrish, Loy & Kline, 1955, 1956) of the use of *Saccharomyces carlsbergensis* 4228 for the assay of the three 'free' forms of vitamin B_6 have appeared since the original observations of Atkin, Schultz, Williams & Frey (1943), the present authors encountered some initial difficulties with the method. In the first instance, of three strains of *S. carlsbergensis* 4228 obtained from various sources, no two had the same nutritional requirements. The strain eventually selected required vitamin B_6 for rapid growth under all conditions of culture tested, so differing from previously used strains of the organism (Rabinowitz & Snell, 1951).

Atkin *et al.* (1943) described a semi-defined medium of pH 5 containing thiamine, upon which their strain of *Saccharomyces carlsbergensis* 4228 grew only slowly in the absence of vitamin B_6; the requirement for vitamin B_6 was later found by Rabinowitz & Snell (1951) to be induced by the presence of thiamine. When incubated in shaken culture at 30° for 18 hr., excellent growth proportional to vitamin B_6 concentrations below 2×10^{-8}M was obtained. Growth of the organism was still proceeding rapidly at the end of incubation, i.e. the rate of growth and not its final extent was dependent upon the vitamin B_6 concentration. The need to ensure that growth did not continue while its extent was being measured was subsequently often emphasized, and various devices, e.g. steaming, refrigeration, addition of chlorothymol, recommended. Inactivity of the phosphorylated derivatives of vitamin B_6 necessitated hydrolytic extraction of natural materials before assay (Atkin *et al.* 1943). Disparity between the activities of the free forms of the vitamin in supporting the growth of various strains of the organism has been described, pyridoxal and pyridoxine usually being equally effective, with pyridoxamine sometimes showing 10–30% less activity (Snell, 1950). It was suggested that supplementation of the basal medium with DL-tryptophan (0·1 mg./ml.) would eradicate this discrepancy (Jones & Morris, 1950). Further addition to the medium of nicotinic acid (2·25 μg./ml.) accelerated growth of the organism in the presence of excess pyridoxine (Hopkins & Pennington, 1947).

Uniform and continuous agitation has commonly been reported to be essential, though it has been stated (without further details) that the assay can be carried out without shaking with 'only a slight decrease in the uniformity of the results' (Snell, 1950), the standing culture tubes being sloped to maintain good aerobic conditions.

Irregularities encountered during the attempted assay of vitamin B_6 according to previously recommended procedures necessitated the detailed study of the response of the selected strain of *Saccharomyces carlsbergensis* 4228 to vitamin B_6; this is the subject of the present paper. The modifications which have been adopted, though apparently slight, have enabled this strain to be used with complete satisfaction in a routine sensitive method for the determination of vitamin B_6 in extracts of various micro-organisms and culture media.

METHODS

Organism. Three cultures all reputed to have been derived from the same parent strain were obtained from different sources. All three were found to possess different nutritional properties. The strain finally selected for use and designated *Saccharomyces carlsbergensis* 4228*c* is no. 534 of the National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey, England. This was chosen as it was dependent upon vitamin B_6 for rapid growth under all conditions of culture tested. The organism was maintained on slopes of medium M stored at 2° after 24 hr. growth at 30°. Stock cultures were subcultured monthly.

Media. Medium M contained (g./l. final vol.), malt extract ('John Bull Brand', Paine and Co. Ltd., St Neots, Hunts), 22·5; ammonium tartrate, 5; yeast extract ('Difco'), 5; sucrose, 5; agar, 20; NH_4NO_3, 1; $MgSO_4.7H_2O$, 0·5; NaCl, 0·1; $CaCl_2$, 0·05; Fe^{2+} (final conc. 2×10^{-5}M) from a solution of $FeSO_4(NH_4)_2SO_4.6H_2O$ in twice its molar concentration of sodium citrate. The pH value was brought to 5·5 with N-NaOH. Medium A ultimately adopted for assays differed from that of Atkin *et al.* (1943) in that it contained nicotinic acid (2·5 mg./l.), and that the amount of acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld, 1945) was decreased to the equivalent of 2 g. casein/l. The pH value was 5–5·2.

All media were prepared at double strength and brought to the desired volume with water and other experimental additions before autoclaving for 7 min. at 115° (unless otherwise stated).

Routine growth tests

(*a*) *Static culture.* Medium (2 ml.) in 150 × 19 mm. hard glass tubes was held in wire baskets sloped at 5° to the horizontal (unless otherwise stated).

(*b*) *Shaken culture.* Medium (5 ml.) was contained in optically matched 150 × 150 × 16 mm. ⊥-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) clamped to a rocking device (similar to that of van Heyningen & Gladstone, 1953) and shaken in a thermostatically controlled water bath at 36 oscillations/min., of excursion 10 cm.

Assessment of growth. The extent of growth was measured with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex, England) with a neutral density filter and 6 mm. sample tubes; the uninoculated medium was used to give the zero setting. The relation between 'EEL reading' and dry weight was determined and was found to be linear up to a reading of 35–40.

RESULTS

Growth characteristics of Saccharomyces carlsbergensis 4228*c*

Growth in static culture. In a preliminary investigation to see whether it would be possible to obtain reproducible growth in static culture, 2 ml. volumes of the basal medium of Atkin *et al.* (1943) were supplemented with pyridoxine (0–10^{-8} M), inoculated with a light suspension of the organism, and incubated at 30° for an arbitrary time of 40 hr. Excellent agreement between duplicate tubes was obtained (Table 1). This was despite the fact that the organisms settled out completely during growth.

Table 1. *Comparison between growth of* Saccharomyces carlsbergensis *in static and shaken culture*

Basal medium of Atkin *et al.* (1943) with pyridoxine as shown, was incubated at 30° for the time stated.

Pyridoxine conc. (M)	Growth (EEL reading)			
	Static 40 hr.		Shaken 16 hr.	
Nil	0	0	0	0
1×10^{-9}	—	—	6·0	6·5
2×10^{-9}	4·5	4·5	12·0	12·0
3×10^{-9}	—	—	18·0	17·5
5×10^{-9}	9·0	9·0	28·0	28·0
7×10^{-9}	—	—	36·5	38·0
1×10^{-8}	14·0	14·5	—	—
$1{\cdot}5 \times 10^{-8}$	20·5	19·5	—	—
2×10^{-8}	25·0	26·0	—	—

Time of incubation. Photometric determinations of growth can be made so rapidly that no special precautions were necessary to prevent the continuance of growth outside the incubator as long as immediate readings were made. The extent of growth obtained with any concentration of the vitamin (below 10^{-8} M) increased with increasing time of incubation (Fig. 1). Measurable growth in the absence of added vitamin B_6 was only obtained by long incubation (100 hr.). To obtain reproducible standard dose-response curves, strict adherence to a selected time of incubation was essential. Even so, it was found necessary to construct a new standard curve in the course of each assay.

Degree of aeration. Good aeration is essential for the rapid growth of this yeast (Atkin *et al.* 1943; Snell, 1950) and most rapid growth was only achieved by constant agitation of the culture (Table 1). If the increased rate of growth under these conditions is a function of the increased aeration and was not due to the dispersal of the organisms then it would be expected that relatively small changes in the degree of aeration might be reflected in the growth response curve. This was found to be the case (Fig. 2) when such changes were produced by alteration of the surface area:volume ratio in static culture. An identical degree of sloping the culture tubes on each occasion of assay was ensured by using standard wire baskets sloped at 5° to the horizontal.

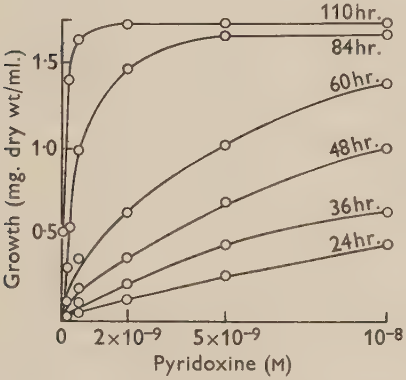

Fig. 1

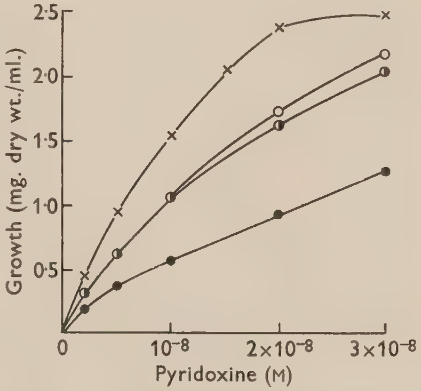

Fig. 2

Fig. 1. Effect of increasing times of incubation on growth response of *Saccharomyces carlsbergensis* 4228*c* to pyridoxine. Basal medium of Atkin *et al.* (1943) supplemented with pyridoxine as shown, was incubated in static culture at 30° for the times indicated.

Fig. 2. Effect of increasing aeration on growth response of *Saccharomyces carlsbergensis* 4228*c* to pyridoxine. Basal medium A (2 ml.) with pyridoxine as shown, incubated for 40 hr. at 30° in variously sloped tubes or 25 ml. conical flasks. Vertical tubes (●), 5° slope (◑) increased slope giving 20 % increased surface area (○), flasks giving greatest surface area/vol. ratio (×).

Temperature of incubation. Though 25° has been suggested as the optimal temperature for growth of *Saccharomyces carlsbergensis* (Parrish *et al.* 1955) strain 4228*c* grows much more rapidly at 36–37° than at 30°, and incubation for 24 hr. at the higher temperature gave a steeper and more linear response over the range of pyridoxine concentrations used (Fig. 3*a*). Further work was carried out at 36–37°, though relatively small changes in incubation time now had a marked effect on the slope of the standard curve (Fig. 3*b*).

Maintenance of a uniform incubation temperature was particularly important when the assay was carried out at 37°, for temperatures only slightly higher (0·5–1°) than this were inhibitory. Although for convenience designated as 37°, the results here reported were obtained in an incubation room whose temperature fluctuated between 36·4° and 37°.

Preparation of the inoculum. To minimize transference of vitamin B_6 from medium M, a small inoculum was used. The organisms were taken from 24 hr.

30° slope culture on medium M; their behaviour did not differ during subsequent incubation in medium A from those harvested after 24 hr. growth at 37° on medium A supplemented with 10^{-7}M pyridoxine. To diminish carry-over still further, the harvested organisms were washed by centrifugation from 0·2 % sterile saline before suspension in fresh saline to a density equivalent to *c.* 4 μg. dry wt./ml. The washed suspension (0·1 ml.) was used to inoculate the volumes (1·9 ml.) of basal medium plus supplements. No growth was obtained in the absence of added vitamin B_6 under the conditions of incubation used. Variation in inoculum size caused a variation in the bulk of growth attained in the standard incubation period with a particular concentration of pyridoxine.

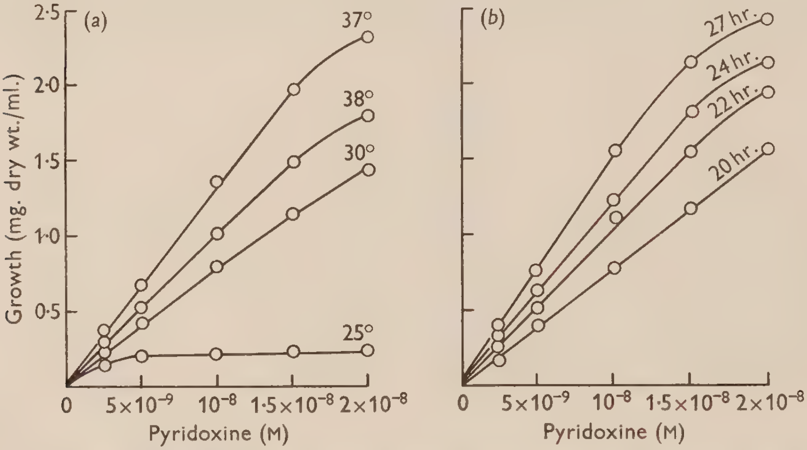

Fig. 3. Growth response of *Saccharomyces carlsbergensis* 4228*c* to pyridoxine after incubation for (*a*) 24 hr. at various temperatures, and (*b*) various times at 37°. Tested in basal medium A in static culture.

Modification of the basal medium. Better growth (20 %) of *Saccharomyces carlsbergensis* 4228*c* was obtained when the concentration of casein hydrolysate in the basal medium was made less than that recommended by Atkin *et al.* (1943), and an amount equivalent to 2 g. casein/l. medium was used. Nicotinic acid slightly increased growth (Fig. 4) and was added to the basal medium in relatively high concentration (2×10^{-5}M). As no growth was obtained (24 hr.) in the absence of vitamin B_6, $(NH_4)_2HPO_4$ previously advised to decrease growth in the blanks (Hopkins & Pennington, 1947), was not used.

Effect of thiamine. Rabinowitz & Snell (1951) reported that the growth of their strain of *Saccharomyces carlsbergensis* 4228 was not dependent upon added vitamin B_6 in the absence of added thiamine. In very small concentrations thiamine caused a growth inhibition which was overcome non-competitively but in a strictly quantitative fashion by pyridoxine. When their inoculum was grown in a thiamine-rich medium, sufficient was carried over endogenously to necessitate adding vitamin B_6 if growth were to be obtained subsequently in a thiamine-free medium. Their organism could therefore only be used for the assay of vitamin B_6 in the presence of an excess of thiamine. The strain used in the present work showed an entirely different thiamine–vitamin B_6 relationship.

In the absence of both vitamins, or of vitamin B_6 only, no growth of our organism was obtained in 24 hr. incubation at 37° (Fig. 4). In the absence of added thiamine, ten times as much pyridoxine was required to support maximal growth as when a basal medium was used containing the concentration of thiamine ($7{\cdot}5 \times 10^{-7}$M) recommended by Atkin *et al.* (1943). Increasing the concentration much above this value had little stimulatory effect (Fig. 4) so that the presence of even considerable concentrations of thiamine in natural extracts will not interfere with the assay of vitamin B_6, when using our strain 4228*c*. Similar results were obtained when this strain was incubated at 30° as in the experiments of Rabinowitz & Snell (1951).

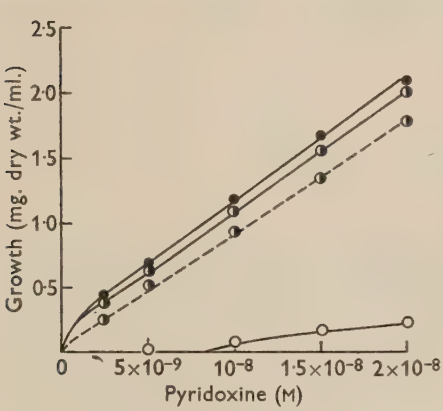

Fig. 4

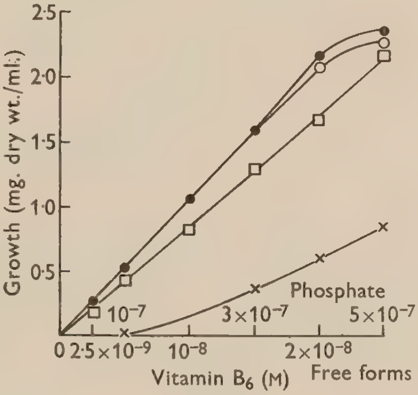

Fig. 5

Fig. 4. Effect of nicotinic acid and thiamine on the growth response of *Saccharomyces carlsbergensis* 4228*c* to pyridoxine. Medium A without nicotinic acid (---), with nicotinic acid, 2×10^{-5}M- (——), without thiamine (○), with $7{\cdot}5 \times 10^{-7}$M-thiamine (◑), with 5×10^{-5}M-thiamine (●).

Fig. 5. Response of *Saccharomyces carlsbergensis* 4228*c* to various forms of vitamin B_6. Medium A supplemented with Seitz-filtered solutions of pyridoxine (●), pyridoxal (○), pyridoxamine (□), and pyridoxal-5′-phosphate (×). Incubated in static culture for 24 hr. at 37°.

Response to various forms of vitamin B_6. Pyridoxal was just as effective as pyridoxine in promoting growth of strain 4228*c* on medium A and the activity of both forms was independent of the pH value of the basal medium within the range pH 3·5 to 5·8. Pyridoxamine appeared to be somewhat less active, but it is uncertain to what extent this was due to partial decomposition of the available sample of pyridoxamine (Fig. 5 and Table 2). Addition of DL-tryptophan (0·1 mg./ml.) had no effect on the response of the organism to pyridoxamine alone, or to an extract of natural material (*Escherichia coli*) known from bioautography to contain pyridoxamine.

Method used for the routine assay of vitamin B_6

Extraction. Hydrolysis of samples of pyridoxal phosphate and pyridoxamine phosphate was complete after autoclaving in 0·055 N-H_2SO_4 at 126° for 2 hr. With all samples of bacteria and growth media used, maximal extraction was always achieved by 2–3 hr. autoclaving.

Table 2. *Growth response of* Saccharomyces carlsbergensis 4228*c* *to various forms of vitamin* B_6

Activity expressed as percentage of growth achieved in medium A with equimolar pyridoxine. Incubation 24 hr. at 37° in static culture.

Form of vitamin B_6	Activity
Pyridoxal	100
Pyridoxamine	80 to 100
Pyridoxal phosphate	2
Pyridoxamine phosphate	< 0·4

The material containing usually 1–10 mμmole vitamin B_6 was diluted to 40 ml. in a 175 × 32 mm. glass tube, acidified with 5 ml. of 0·5 N-H_2SO_4 and autoclaved for 3 hr. at 126°. Alternatively, 10 ml. samples + 1·25 ml. 0·5 N-H_2SO_4 were autoclaved in 150 × 19 mm. tubes with no loss of efficiency. After extraction, a sample was brought to pH 5 with a measured volume of N-NaOH (bromocresol green as external indicator), clarified by centrifugation and usually assayed immediately. When stored at 2° for any length of time before assay, the extracts were kept in the dark.

When using heavily buffered samples (e.g. of growth media) it was necessary to add more acid to bring the pH value to the required range of 1·6–2·0 (Rubin, Scheiner & Hirschberg, 1947). With most of the culture media tested, 40 ml. samples required the addition of approximately 8 ml. of 0·5 N-H_2SO_4.

Assay procedure. The samples or appropriate dilutions, at four different concentrations within the assay range, were added in 0·1–0·8 ml. quantities to 150 × 19 mm. tubes containing 1 ml. double strength medium A. Standards containing 5, 10, 20, 30, 40 and 50 $\mu\mu$mole pyridoxine were included in each complete assay. After dilution to 1·9 ml. and autoclaving at 10 lb. sq.in. for 7 min., the tubes were inoculated with 0·1 ml. of a suspension containing equiv. *c.* 4 μg. dry wt./ml. of *Saccharomyces carlsbergensis* 4228*c*. This was prepared by suspending organisms harvested from a 24 hr. culture on medium M in sterile 0·2 % saline, centrifuging and resuspending in fresh saline to the required density. Incubation was for 24 hr. at 36–37° with the tubes sloped at 5° to the horizontal. Growth was assessed immediately on removal of the cultures from the incubator.

The useful range of the assay was from 5–40 $\mu\mu$mole pyridoxine corresponding to growth equiv. 0·2–2·0 mg. dry wt. yeast/ml. All tubes were set up in duplicate and exposed as little as possible to direct illumination.

Validity of assay of bacterial extracts. Extracts of various bacterial cultures might interfere with the assay of vitamin B_6 by stimulating or inhibiting growth of *Saccharomyces carlsbergensis* 4228*c* in vitamin B_6-supplemented medium A. It was found with all extracts tested that: (*a*) the dose-response curves relating concentration of extract and resultant growth of the yeast were superimposable upon the standard curve obtained with pyridoxine; (*b*) the response curves obtained when a known concentration of pyridoxine was added to various concentrations of extract, and conversely, when a small amount of extract was added to each of the standard tubes containing known concentrations of pyridoxine, both lay parallel to the standard curve; (*c*) pyridoxine added to the extract was quantitatively recovered (see below). Typical results obtained with an extract of an *Escherichia coli* mutant are given in Fig. 6. It is recommended that such control experiments be carried out upon each new material to be assayed.

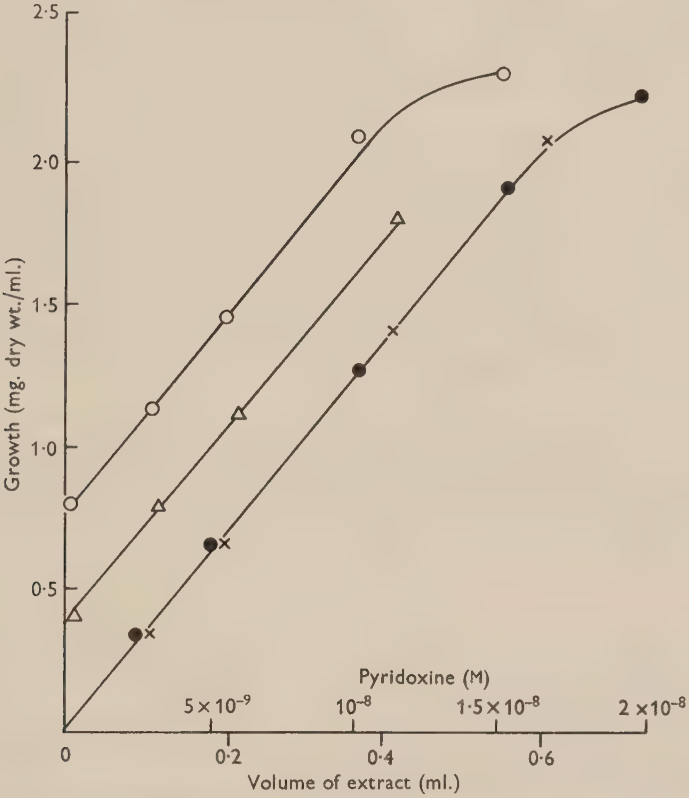

Fig. 6. Assay of vitamin B_6 content of extract of *Escherichia coli* B166. Growth of *Saccharomyces carlsbergensis* 4228*c* measured after 24 hr. static incubation at 37° in medium A with amounts of pyridoxine (●), extract (×), extract + 3×10^{-9} M pyridoxine (△), and pyridoxine + 0·2 ml. extract (○).

Accuracy. The recovery of pyridoxine added to bacterial extracts was 100 % ± 10 %. When results obtained by this method, but in another laboratory, were subjected to statistical analysis, the S.D. was found to be 8·2 % (Dr Joan F. Powell, Porton; personal communication).

DISCUSSION

It is recommended that the growth requirements of any strain of *Saccharomyces carlsbergensis* 4228 be well studied before it is used as an assay organism, for various strains differ markedly from each other, particularly in their response to thiamine and vitamin B_6. Thiamine must be present in excess when vitamin B_6 is assayed with the strain used by Rabinowitz & Snell (1951) or with that (4228*c*) described above, but for quite different reasons. With the first strain thiamine produces the requirement for vitamin B_6, with the second, thiamine diminishes the amount of pyridoxine needed for any given rate of growth.

As emphasized throughout, the fact that the method is a 'rate assay' exaggerates the need for strict adherence to the chosen test conditions, but it has the useful corollary that within limits the sensitivity of the assay can be adjusted by varying the time of incubation. Thus by extending the incubation period beyond 24 hr. at 37°, it is possible to assay with accuracy over a range of pyridoxine concentrations lower than 5×10^{-9} M. Growth in the complete absence of pyridoxine, i.e. in the 'blank', becomes progressively greater with these longer times of incubation.

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REFERENCES

ATKIN, L., SCHULTZ, A. A., WILLIAMS, W. L. & FREY, C. N. (1943). Yeast microbiological methods for determination of vitamins. Pyridoxine. *Industr. Engng Chem. (Anal.)*, **15**, 141.

FOURNIER, S. A., THOMAS, M. H. & SEIGEL, G. L. (1951). Vitamin B_6. In *Methods of Vitamin Assay*, 2nd ed., p. 217. Ed. The Association of Vitamin Chemists Inc. New York: Interscience Publishers, Inc.

HEYNINGEN, W. E. VAN & GLADSTONE, G. P. (1953). The neurotoxin of *Shigella shigae*. III. The effect of iron on production of the toxin. *Brit. J. exp. Path.* **34**, 221.

HOPKINS, R. H. & PENNINGTON, R. J. (1947). The assay of the vitamin B_6 complex. *Biochem. J.* **41**, 110.

JONES, A. & MORRIS, S. (1950). A 24-hour plate assay technique for the vitamin B_6 complex of yeast. *Analyst*, **75**, 613.

MONOD, J., COHEN-BAZIRE, G. & COHN, M. (1951). Sur la biosynthese de la β-galactosidase (lactase) chez *Escherichia coli*. La spécificité de l'induction. *Biochim. biophys. Acta*, **7**, 585.

PARRISH, W. P., LOY, H. W., JR. & KLINE, O. L. (1955). A study of the yeast method for vitamin B_6. *J. Ass. off. agric. Chem., Wash.* **38**, 506.

PARRISH, W. P., LOY, H. W., JR. & KLINE, O. L. (1956). Further studies on the yeast method for vitamin B_6. *J. Ass. off. agric. Chem., Wash.* **39**, 157.

RABINOWITZ, J. C. & SNELL, E. E. (1951). The nature of the requirement of *Saccharomyces carlsbergensis* for vitamin B_6. *Arch. Biochem. Biophys.* **33**, 472.

RUBIN, S. H., SCHEINER, J. & HIRSCHBERG, E. (1947). The availability of vitamin B_6 in yeast and liver for growth of *Saccharomyces carlsbergensis*. *J. biol. Chem.* **167**, 599.

SNELL, E. E. (1950). Vitamin B_6. In *Vitamin methods*, **1**, 438. Ed. Gyorgy, P. New York: Academic Press Inc.

SNELL, E. E. & RANNEFELD, A. N. (1945). The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. *J. biol. Chem.* **157**, 475.

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Morris, J. G. & Woods, D. D. (1959). *J. gen. Microbiol.* **20**, 576–596

Inter-relationships of Serine, Glycine and Vitamin B_6 in the Growth of Mutants of *Escherichia coli*

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SUMMARY: Five mutants of *Escherichia coli* which grow to a greater or less extent when the minimal medium is supplemented with vitamin B_6 have been studied. They fall into two groups according to the further effect of serine + glycine on growth. (1) Two strains showed full and rapid growth with pyridoxine alone and serine + glycine neither replaced this vitamin nor enhanced growth in its presence. (2) Three strains showed delayed and suboptimal growth with pyridoxine alone. With these serine + glycine had two effects: (*a*) it decreased the lag and enhanced the final extent of growth when added with pyridoxine; (*b*) it permitted suboptimal growth in the absence of pyridoxine. The second effect was dependent on (two strains), or greatly increased by (one strain), the concurrent addition of glycolaldehyde.

Nothing is known of the pathway of synthesis of vitamin B_6 by organisms not requiring an external source of it for growth. Mutant strains of *Escherichia coli* which multiply only when vitamin B_6 is supplied are more rare than those which respond to vitamin B_6 or serine or glycine. The simplest explanations of the apparent replacement of the vitamin by these amino acids are: (*a*) the amino acids are end products of reactions catalysed by vitamin B_6 derivatives; consequently, when provided preformed, they decrease or abolish the need for the vitamin; (*b*) the synthesis of serine and glycine by the organism is limited, and one or other is a reactant at some step in the biosynthesis of vitamin B_6.

A detailed study was made of the properties of mutants of this type in an attempt to discover an immediate biochemical lesion resulting from the mutation and possibly to obtain some conception of the route of synthesis of vitamin B_6 and its derivatives by normal strains of *Escherichia coli.*

METHODS

Escherichia coli. Stock cultures were maintained on tryptic meat agar slopes and were subcultured monthly (16 hr. at 37°). Strains B and 15 (American Type Culture Collection, ATCC no. 9723) were prototrophs. Strains B166 and B167 (obtained from Dr J. Gots and derived from strain B), and 22-99 (obtained from Professor B. D. Davis) required vitamin B_6 or serine or glycine for growth. Two strains responded only to vitamin B_6. These were: strain M2, which required the vitamin at all temperatures and was isolated by us by the technique of Adelberg & Myers (1953) after u.v. irradiation of strain 15; strain DW, a substrain of M154-59L, provided by Dr Elizabeth Work (Denman, Hoare & Work, 1955) which required vitamin B_6 for growth at 37° but responded also to certain mixtures of amino acids at lower temperatures.

Strain B166 was used for most of the work to be described.

Media

Based on glucose. Medium G1 was that of Davis & Mingioli (1950) and medium G2 the same but with the glucose concentration increased to 1% (w/v). The glucose when not autoclaved separately in aqueous solution (as in media G1 and G2) was sterilized as indicated in individual experiments.

Based on lactate. Medium L contained (g./l.): KH_2PO_4, 2; Na_2HPO_4, $12H_2O$, 8; $(NH_4)_2SO_4$, 2·5; NH_4Cl, 2·5; lactic acid, 10; $MgSO_4,7H_2O$, 0·05. After addition of Fe^{2+} (final conc. 4×10^{-6}M) from a solution of $FeSO_4.(NH_4)_2SO_4,6H_2O$ in twice its molar concentration of sodium citrate, the pH value was brought to 7·4 with 10N-NaOH.

All media were prepared at double strength and brought to the desired volume with water and other experimental additions before autoclaving for 7 min. at 115° (unless otherwise stated).

Supplements. Amino acid mixture AA-SG contained the following amino acids at 5×10^{-3}M: DL-alanine, L-arginine, DL-aspartic acid, L-cysteine HCl, DL-glutamic acid, DL-histidine, L-hydroxyproline, DL-isoleucine, DL-leucine, DL-lysine.diHCl, DL-methionine, DL-phenylalanine, DL-proline, DL-threonine, DL-tryptophan, DL-tyrosine, DL-valine.

The purines + pyrimidines mixture contained 2×10^{-4}M adenine, and 10^{-4}M each of guanine, uracil, thymine and hypoxanthine. Addition of a little N-HCl facilitated solution (later neutralized).

Vitamin mixture V contained (μg./ml.): riboflavin, 50; thiamine, 60; calcium pantothenate, 60; nicotinic acid, 100; biotin, 0·1; *p*-aminobenzoic acid, 1·4; cobalamin, 0·1.

Growth conditions

Static culture. Inoculated medium (5 ml.) in 150 × 19 mm. hard glass tubes were incubated at 37° in racks sloped at 5° to the horizontal. The inoculum was 0·1 ml. of aqueous suspension of organisms (about $2 \cdot 5 \times 10^7$/ml.) derived from a 24 hr. culture on tryptic meat agar.

Shaken culture. Medium (5 ml.) inoculated as above was contained in optically matched 150 × 150 × 16 mm. ⊥-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) which were clamped to a rocking device (similar to that of van Heyningen & Gladstone, 1953) and shaken in a 37° water bath at 36 oscillations/min., of excursion 10 cm.

When larger quantities of organisms were required, Erlenmeyer flasks (1 l.) containing 500 ml. medium were inoculated with about $2 \cdot 5 \times 10^8$ organisms and shaken at 37° (circular motion, 220 rotations/min. of radius 2 cm.).

Assessment of growth

The extent of growth was measured with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex, England) with a neutral density filter; the uninoculated medium was used to give the zero setting. The cross arms of the 16 mm. ⊥-tubes were constructed to fit the instrument; samples of other cultures were assessed in 6 mm. tubes. A curve relating the readings in the two types of tubes was constructed, and the growth achieved by the organisms under all conditions of incubation expressed in terms of the EEL reading which would have been obtained in ⊥-tubes. The relation between instrument reading and dry weight was determined for each strain used and was found to be linear up to a reading of 35–40; a reading of 20 was equivalent to 0·6 mg. dry wt./ml.

Estimation of glycolaldehyde

The colorimetric method of Dische & Borenfreund (1949) was used. Trichloroacetic and glacial acetic acids were of AR grade and the diphenylamine recrystallized twice from 70% (v/v) ethanol before immediate use. The colour developed was measured by reading optical densities at 660 and 580 mμ. The method was stated to permit the determination of glycolaldehyde in the presence of a fifty-fold excess of trioses and other aldehydes and sugars. In the present work, no aldehyde or other chemically related compound tested at concentrations within this range was found to interfere appreciably with the assay of glycolaldehyde.

A standard curve relating D_{660}–D_{580} to glycolaldehyde concentration over the range 2×10^{-4} to 10^{-3}M was made in each assay. The relationship was always linear.

Chemicals

Glycolaldehyde. Commercial preparations were normally used, but for critical tests one sample was recrystallized from absolute ethanol. A further pure specimen (m.p. 94·5–95°) was prepared from tartaric acid via dihydroxyfumaric acid (Powers, Tabakoglu & Sable, 1955) and recrystallized from dry acetone. Glycolaldehyde-2-phosphate (barium salt) was kindly provided by Dr E. Racker and converted to the sodium salt as described below.

Other chemicals. A solution of β-hydroxypyruvic acid was obtained by the controlled alkaline hydrolysis of β-bromopyruvic acid (Sprinson & Chargaff, 1946). Methylglyoxal in solution was prepared from dihydroxyacetone (Umbreit, Burris & Stauffer, 1945) and assayed by Friedemann's (1927) method. Glucose reductone (*enol*tartronaldehyde) was made from glucose (von Euler & Martius, 1933).

Xylulose-5-phosphate, sedoheptulose-7-phosphate (as barium salts) and the *o*-nitrophenylhydrazone of L-erythrulose were gifts from Dr B. L. Horecker. Sodium salts (in solution) were obtained by batch treatment with Amberlite IR 50 resin in the Na$^+$ form, while a solution of free L-erythrulose was prepared by a small-scale adaptation of the method of Müller, Montigel & Reichstein (1937). A solution of D-erythrose-4-phosphate was obtained (Ballou, Fischer & McDonald, 1955) from a specimen of the cyclohexylamine salt of the acetal donated by Dr C. E. Ballou. δ-Aminolaevulic acid was given by Professor A. Neuberger. All other chemicals were commercial specimens.

RESULTS

Except in the final section, the experiments were all performed with *Escherichia coli* B166 which grows on certain media with added glycine or serine or vitamin B_6. For reasons explained later the amino acids were used, unless otherwise stated, as an equimolar mixture of DL-serine and glycine; this will be referred to as serine + glycine, the molar concentration given being in terms of the total amino-nitrogen. Serine and glycine are known to be metabolically very closely related in *E. coli* (Cutinelli *et al.* 1951; Abelson, 1954; Meinhart & Simmonds, 1955), and as they were essentially interchangeable in the present study, their use in admixture introduces no difficulties of interpretation. Vitamin B_6 is used in general reference to the group; individual members will be specifically named.

General characteristics of growth

Comparison of glucose and lactate as main carbon source. The response of strain B166 to pyridoxine (10^{-6} M), serine + glycine (10^{-3} M) and mixtures, was tested on the simple glucose-containing medium G1, and the ammonium lactate medium L (Figs. 1 and 2). The main carbon source was added to each of the media before autoclaving.

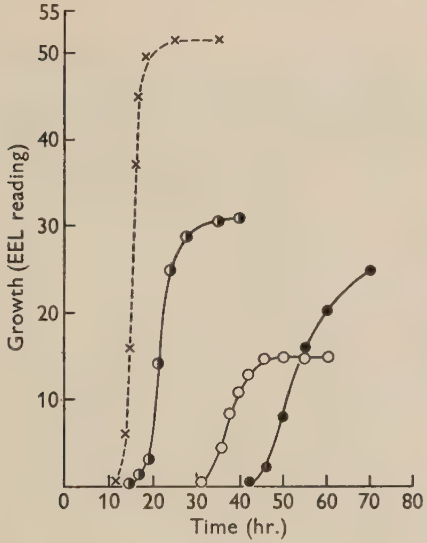

Fig. 1

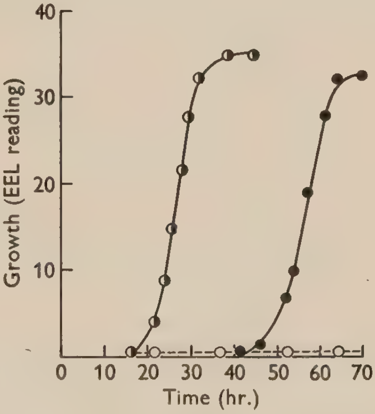

Fig. 2

Fig. 1. Growth of *Escherichia coli* B166 on medium G1 (with glucose autoclaved in the medium). Basal medium was supplemented with serine + glycine, 10^{-3} M (○), pyridoxine, 10^{-6} M (●), serine + glycine and pyridoxine (◑); growth on unsupplemented medium of prototrophic parent strain *Escherichia coli* B (–×–). Incubated in shaken culture at 37°.

Fig. 2. Growth of *Escherichia coli* B166 on lactate medium L. Basal medium was supplemented with serine + glycine, 10^{-3} M (○), pyridoxine, 10^{-6} M (●), serine + glycine and pyridoxine (◑). Incubated in shaken culture at 37°.

A notable difference was that serine + glycine did not support growth on the lactate medium. In both media, however, most rapid and extensive growth occurred with pyridoxine plus serine + glycine, the rate then approaching that of the parent strain growing on unsupplemented medium. With pyridoxine alone, growth was delayed in both media but the final mass was 80 to 90 % of that obtained with the mixture. In all cases where growth occurred on lactate medium it was slightly delayed, but finally 10–20 % greater, than with the same supplement in the glucose medium.

In glucose medium supplemented only with serine + glycine, growth was delayed less than with pyridoxine alone, but the final mass was only 50–60 % of that found with both supplements (Fig. 1). The mixture of serine and glycine had therefore two apparently distinct effects on growth; (*a*) it supported growth in the absence of pyridoxine but only on the glucose medium, while (*b*) on both media, and with pyridoxine present, it decreased the delay in onset of growth by 20–25 hr. and slightly increased the final density.

Response to vitamin B_6. With the five forms of vitamin B_6 tested on medium G1 (Table 1) growth was most rapid with pyridoxine; the concentration for half-maximal growth was 2×10^{-7}M. Pyridoxal was intrinsically more active (10^{-7}M gave half-maximal response) but growth was delayed a further 12 hr. Similar results were obtained on medium L. Pyridoxine was subsequently used routinely.

Table 1. *Response of* Escherichia coli B166 *to various forms of vitamin* B_6

Basal medium G1 with glucose autoclaved in the medium at 115°/7 min. Incubation in static culture at 37°; tubes containing serine + glycine incubated for 30 hr. only, the remainder for 80 hr.

Supplement	Conc. (M)	Growth (EEL reading) with serine + glycine (10^{-3}M) Absent	Present
Pyridoxine	10^{-6}	14	18
	3×10^{-7}	11	18
	10^{-7}	4	10
	3×10^{-8}	0	6
	10^{-8}	0	0
Pyridoxal	10^{-6}	12	17
Pyridoxamine	10^{-6}	6	14
Pyridoxal-5′-phosphate	10^{-6}	5	—
Pyridoxamine-5′-phosphate	10^{-6}	0	—

With serine + glycine also present (Table 1) the amount of pyridoxine required was decreased to about one-third (6×10^{-8}M for half-maximal growth); in these experiments results were read at 30 hr. since on longer incubation there was of course growth in the absence of added pyridoxine.

Response to serine, glycine and related compounds. The concentration of serine + glycine required on medium G1 (glucose added before autoclaving and no added pyridoxine) was 10^{-3} and $2 \cdot 5 \times 10^{-4}$M for maximal and half-maximal growth respectively (Table 2). Similar concentrations were required for the other effect of serine + glycine, i.e. to promote earlier growth when pyridoxine was present. Glycine and DL-serine were individually active in both the above senses, but the mixture gave somewhat more rapid and heavier growth. In addition, results were erratic with suboptimal concentrations of the separate amino acids. This appeared to be due to partial reversion of the

Table 2. *Amino acid growth requirements of* Escherichia coli B166 *in the absence of added vitamin* B_6

Incubation for 70 hr. at 37°. Other conditions as in Table 1.

Amino acid	Conc. (M)	Growth (EEL reading)
Nil	—	0
DL-Serine + glycine	4×10^{-3}	12
	10^{-3}	12
	5×10^{-4}	9
	$2\cdot5 \times 10^{-4}$	6
	$1\cdot25 \times 10^{-4}$	4
	$6\cdot25 \times 10^{-5}$	3
L-Serine	5×10^{-4}	7
D-Serine	5×10^{-4}	0
Glycine	5×10^{-4}	8
DL-Homoserine	10^{-3}	0
DL-Threonine	10^{-3}	Trace*

* 5 after longer incubation (110 hr.).

mutant to prototroph-like character. Random individual tubes grew heavily after 48 hr. incubation and subcultures into basal medium gave growth (though less rapidly than the parent strain) without the addition of either pyridoxine or serine + glycine. The properties of these substrains have not been further pursued. These changes were less frequent with mixtures of glycine and serine at suboptimal concentration and did not occur when the concentration of the individual amino acids exceeded 4×10^{-4}M. With serine alone (at optimal concentration) only the L-isomer was active, either in the presence or absence of pyridoxine.

There is evidence that *Escherichia coli* can form glycine from both threonine and homoserine (Gibson, 1952; Abelson, 1954). DL-Threonine at high concentration supported detectable growth in the absence of pyridoxine but DL-homoserine was inactive (Table 2).

Other amino acids or substances metabolically related to serine and glycine and found to be inactive (over a concentration range of 10^{-6} to 5×10^{-3}M) were: formiminoglycine, glycollic, glyoxylic, pyruvic, β-hydroxypyruvic and δ-aminolaevulic acids, ethanolamine, DL-alanine, L-arginine, DL-asparagine, DL-aspartic acid, DL-glutamic acid, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-phenylalanine, DL-tryptophan, DL-tyrosine and DL-valine.

Effect of autoclaved glucose. The fact that serine + glycine supported growth of strain B166 in the absence of pyridoxine, but only on the glucose-containing medium, suggested that the activity of the amino acids was dependent on the concurrent presence of some substance formed as a result either of glucose metabolism by the organism or of the heat-sterilization of the medium. In the above experiments, the glucose had been added to the other constituents of the medium before autoclaving. When the glucose was separately sterilized by Seitz-filtration, and then added to the remainder of the medium (which had been autoclaved as usual), serine + glycine no longer supported growth. This was also the case when the glucose was separately sterilized by autoclaving in aqueous solution (in glass-distilled water when the pH was 5·4; Fig. 3). Autoclaving in 0·04 M-phosphate buffers of various pH values showed that only at about pH 7 and above was a solution of glucose obtained which permitted growth on serine + glycine (Fig. 3).

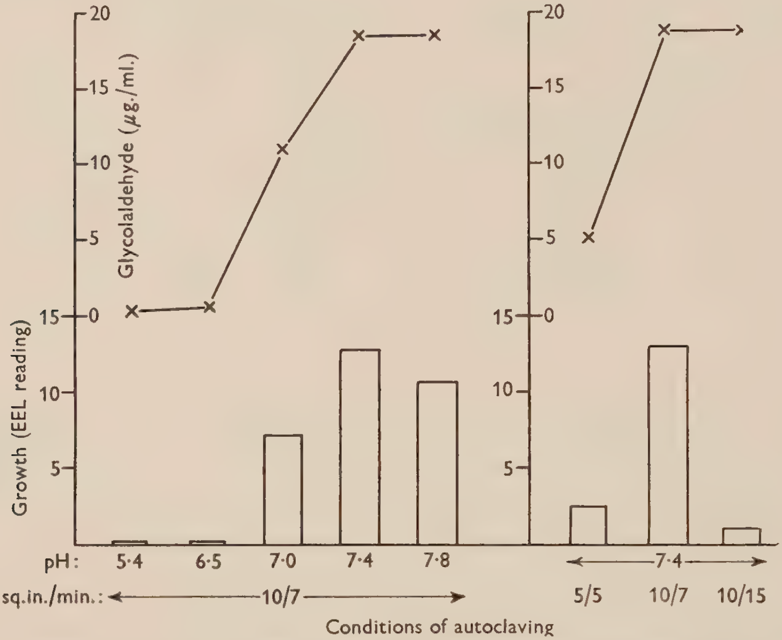

Fig. 3. Comparison of growth promoting activity of heated glucose solutions (block diagram) with their content of substance assaying as glycolaldehyde (–×–). Activated glucose samples were prepared by autoclaving glucose solutions (10 mg./ml.) in 0·04 M-phosphate buffers. Activity measured as extent of growth of *Escherichia coli* B166 in static culture on medium G2 supplemented with sample plus serine + glycine (10^{-3} M).

For present purposes, the term 'activated glucose' will be used in reference to glucose solutions which permit growth of the organism on serine + glycine without added pyridoxine. 'Inactive glucose' will be used in the opposite sense. Untreated glucose was inactive, and remained so after sterilization by Seitz-filtration or by autoclaving at pH 6·5 and below.

The action of activated glucose

Conditions for formation. Glucose (10 mg./ml.) was autoclaved at various combinations of temperature and time, at pH values ranging from 6 to 8. After neutralizing, the solution was added to previously sterilized basal medium supplemented only with serine + glycine. Best activation occurred when autoclaving was carried out at 10 lb. sq. in./7 min. at pH 7·4 (Fig. 3). At higher pH values or with more severe autoclaving, there was obvious caramelization and a decrease in activity, probably due rather to the concurrent production of inhibitory substances than to decreased formation of the active factor. Onset of growth in the presence of serine + glycine plus pyridoxine was delayed 10, 10 and 30 hr., respectively, when the glucose was treated at pH 7·8 (10 lb. sq. in./7 min.) or at pH 7·4 for 15 min. at 10 and 15 lb. sq. in.

Circumstances when required. As shown above both serine + glycine and activated glucose were required for growth in the absence of pyridoxine. Experiments with mixtures of inactive and activated glucose showed that the maximum effect was obtained when at least 80 % of the glucose in the medium was provided in the activated form.

When pyridoxine was present (either with or without serine + glycine), activated glucose was not required, nor did it increase the rate or final extent of growth (Fig. 4). The only effect was a slight delay in the onset of growth, possibly again due to the presence of inhibitory substances. Activated glucose was therefore not required for the second action of serine + glycine (i.e. decreasing the lag when pyridoxine is present), but only for the total replacement of pyridoxine.

Table 3. *Reducing agents and the growth of* Escherichia coli B166 *on serine + glycine*

The reducing agents were sterilized by Seitz-filtration. Incubated in static culture 64 hr. at 37°.

		Growth (EEL reading) with serine + glycine (10^{-3} M)	
Supplement	Conc. (M)	Alone	+ Pyridoxine (10^{-6} M)
Nil	—	0	15
Ascorbic acid	2×10^{-3}	0	0
	4×10^{-4}	0	(15)
	8×10^{-5}	0	15
Glucose reductone	10^{-5}	0	(15)
*Activated glucose	5×10^{-2}	10	15

( ) Signifies growth delayed 10 hr. compared with control.

* Control in which glucose was added to basal medium G1 before autoclaving.

Effect of reducing agents. The activation of glucose might simply have been due to the development of non-specific reducing conditions in the autoclaved mixtures. One powerful reducing agent known to be formed when alkaline solutions of glucose are heated is glucose reductone (*enol*tartronaldehyde; Pigmann & Goepp, 1948). This compound (10^{-10} to 10^{-5}M) did not replace activated glucose for growth of strain B166 on serine + glycine; at concentrations above 10^{-5}M it partly inhibited growth in the controls (Table 3). It may be partially responsible for the slight delay in growth obtained when activated glucose was present in the medium. Ascorbic acid was chosen as a reducing agent of carbohydrate-like nature, though one which is not formed from glucose on autoclaving. It, too, failed to replace activated glucose at the concentrations tested (10^{-8} to 5×10^{-3}M) and was inhibitory at the higher concentrations (Table 3).

Activated glucose was also necessary for growth on serine + glycine under anaerobic conditions (Table 8). It was concluded that the activation of glucose is due to the formation of some specific compound which together with serine + glycine permits growth of strain B166 in the absence of added vitamin B_6.

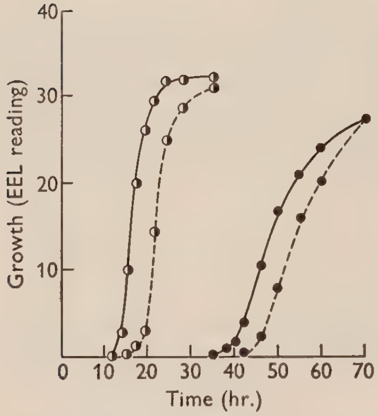

Fig. 4

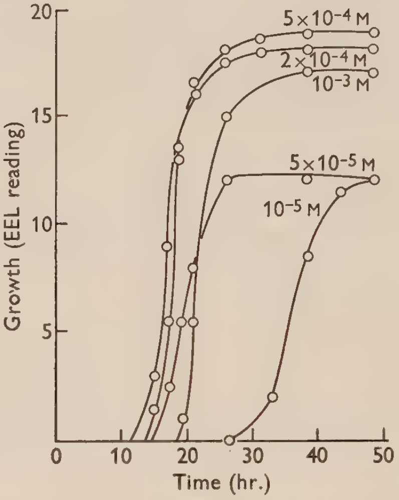

Fig. 5

Fig. 4. Effects of (*a*) inactive glucose (——) and (*b*) activated glucose (– – –) on growth of *Escherichia coli* B166 in medium G1 supplemented with pyridoxine, 10^{-6} M, alone (●) and with serine + glycine 10^{-3} M (◑). Incubated in shaken culture at 37°.

Fig. 5. Growth of *Escherichia coli* B166 on medium G2 supplemented with serine + glycine 10^{-3} M and indicated concentrations of glycolaldehyde. Incubated in shaken culture at 37°.

Experiments with other sugars and derivatives. Material active in supporting growth in a medium supplemented only with serine + glycine was also formed on autoclaving certain other hexoses and pentoses at slightly alkaline pH values.

Solutions (20 mg./ml.) were (*a*) Seitz-filtered, (*b*) autoclaved at the existing pH (5·4–5·7), (*c*) autoclaved in 0·04 M phosphate buffer pH 7·4, and (*d*) added to the supplemented basal medium (pH 7) before autoclaving. The treated solutions were tested at a final concentration of 4 mg./ml. The additional carbohydrate did not affect the rate or extent of growth in control media supplemented with pyridoxine (with or without serine + glycine).

None of the sugars permitted growth with serine + glycine unless they had first been autoclaved at pH 7 or 7·4 (Table 4). The three sugar alcohols and 3-O-methylglucose remained inactive even after such treatment. Fructose gave rise most easily to inhibitory substances, and the growth which did occur on serine + glycine with autoclaved preparations of fructose was delayed 24–48 hr. as compared with the other sugars.

Several of the carbohydrates are fermented by *Escherichia coli* and could replace glucose as the main carbon source for growth of the mutant in the presence of pyridoxine. In these cases, development of activity was also assayed by adding the carbohydrate to the medium (i.e. G1 without glucose) as the sole carbon source either before autoclaving or as a Seitz-filtered solution after autoclaving. The sugar alcohol mannitol was inactive in both cases; the sugars, as before, were active only after autoclaving (Table 5).

Table 4. *Ability of variously treated sugars and their derivatives to replace activated glucose*

Criterion of activity was growth of *Escherichia coli* B166 on medium G2 supplemented with serine+glycine (10^{-3} M) plus the test compound. All compounds under test were used at a final concn. of 4 mg./ml. and autoclaved (115°/7 min.) as shown at 20 mg./ml.

		Autoclaved in		
Compound	Seitz-filtered	Aqueous soln. (pH 5·4–5·6)	Medium (pH 7)	PO_4 buffer (pH 7·4)
Galactose	—	—	+	+
Mannose	—	—	+	+
Fructose*	—	—	+	+
Sorbose	—	—	+	+
Arabinose	—	—	+	+
Xylose	—	—	+	+
Ribose	—	—	+	+
Mannitol	—	—	—	—
Sorbitol	—	—	—	—
Erythritol	—	—	—	—
3-O-Methyl-glucose	—	—	—	—

+ = Activation; – = no activation.
* Activated fructose was somewhat inhibitory (delayed growth).

Table 5. *Growth of* Escherichia coli B166 *in the presence of serine+glycine plus variously treated sugars*

Basal medium G1, but with compound specified replacing glucose, plus serine+glycine (10^{-3} M); all autoclaving was at 115°/7 min. Incubation in static culture for 80 hr. at 37°.

		Autoclaved in		
Compound	Seitz-filtered	Aqueous soln. (pH 5·4–5·6)	Medium (pH 7)	PO_4 buffer (pH 7·4)
Galactose	—	—	+	+
Mannose	—	—	+	+
Fructose*	—	—	+	+
Arabinose	—	—	+	+
Xylose	—	—	+	+
Mannitol	—	—	—	—

+ = Growth; — = no growth.
All the above substrates supported growth in the presence of pyridoxine.
* Activated fructose was somewhat inhibitory (delayed growth even in the presence of pyridoxine).

With the relatively small number of carbohydrates tested it would appear that the active substance was formed only when there was an actual or potential aldehyde group at C-1 and an unsubstituted hydroxyl group on C-3.

Behaviour of known degradation products of glucose. Concurrently with the above experiments, a number of compounds reported to be amongst the products of alkaline degradation of glucose (Evans, 1942; Pigmann & Goepp, 1948) were assayed for activity. These were added in Seitz-filtered solution over a range of concentrations (10^{-6} to 5×10^{-3} M), to medium G2 containing serine + glycine.

No growth was obtained with the following substances: lactic, pyruvic, acetic, formic and tartronic acids, methylglyoxal, dihydroxyacetone, DL-glyceraldehyde and formaldehyde. Also tested and found to be inactive was 5-hydroxymethylfurfural (which in any event is predominantly a product of the acidic degradation of glucose).

Glycolaldehyde (5×10^{-5} to 10^{-3} M) supported good growth in the presence of serine + glycine and absence of added pyridoxine. A recrystallized commercial specimen and material prepared from dihydroxyfumaric acid synthesized in this laboratory and recrystallized to m.p. 94·5–95°, were both quantitatively as active as the commercial product which was used in all subsequent experiments.

Further experiments with glycolaldehyde

Media containing glucose. The ability of glycolaldehyde to support growth of *Escherichia coli* B166 on medium G2 supplemented with serine + glycine was studied in aerobic, shaken culture (Fig. 5). Delayed and suboptimal growth was obtained with a glycolaldehyde concentration of 10^{-5} M; 5×10^{-4} M supported greatest growth. Above these concentrations, progressive retardation and diminution of growth was noted until at 5×10^{-3} M it was completely inhibited (even in the presence of pyridoxine). Glycolaldehyde (5×10^{-4} M) was more effective than activated glucose in supporting growth on serine + glycine (Fig. 5, compare Fig. 1), the delay in growth being decreased from 30 to 15 hr. The presence of glycolaldehyde, however, had no effect on growth of the organism in the presence of pyridoxine, altering neither the lag period nor the final yield of organisms (Fig. 6, compare Fig. 4).

Although final growth was better in shaken than in stationary culture under all conditions, the effect of aeration was relatively greater for growth on serine + glycine. Thus the final mass on serine + glycine and glycolaldehyde in shaken tubes was 65–70 % of that with pyridoxine also present but only 50–55 % in stationary tubes (from data of Fig. 6 and Table 7). The optimal glycolaldehyde requirement for growth in stationary culture was somewhat higher (10^{-3} M) than in shaken culture.

Lactate medium. Escherichia coli B166 did not respond to serine + glycine alone in medium L (Fig. 2). Addition of activated glucose permitted limited growth; the concentration of activated glucose required being approximately the same as with the wholly glucose-containing basal medium. Glycolaldehyde (5×10^{-4} M) again replaced activated glucose, allowing suboptimal growth to occur with no greater delay than when pyridoxine was also present (Fig. 7). The final growth was not improved by increasing the concentration of glycolaldehyde; indeed, this was somewhat toxic at a concentration (10^{-3} M) which gave good growth on medium G2. The response in lactate medium to pyridoxine alone, or together with serine + glycine, was not affected by glycolaldehyde (Fig. 7, compare Fig. 2).

Whilst 5×10^{-4}M-glycolaldehyde bettered the action of activated glucose in the glucose medium, it was less effective in that based on lactate (Fig. 7). When inactive glucose (10 mg./ml.) was added with the glycolaldehyde, there was no effect on the ultimate mass achieved in the presence of pyridoxine, but the suboptimal growth obtained in the absence of added vitamin was doubled (Fig. 8, compare Fig. 7). Galactose, gluconate and fructose could be substituted for glucose, but other possible carbon sources, e.g. arabinose, ribose, pyruvate, glyceraldehyde and acetate, were inactive or much less active. Addition of glycolaldehyde alone to either glucose or lactate medium did not allow of growth of the organism or reduce its pyridoxine requirement.

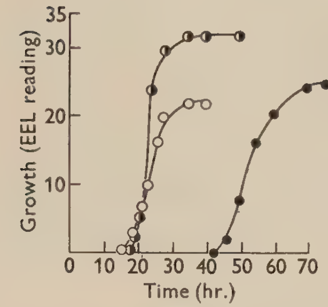

Fig. 6

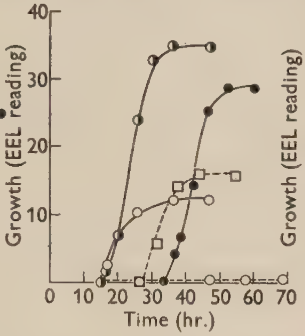

Fig. 7

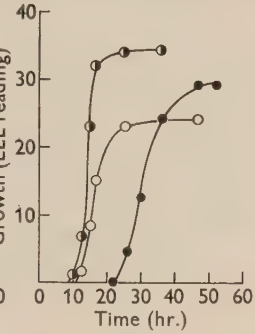

Fig. 8

Fig. 6. Growth of *Escherichia coli* B166 on medium G2 supplemented with glycolaldehyde (5×10^{-4}M). Other additions were: serine + glycine 10^{-3}M (○), pyridoxine, 10^{-6}M (●), serine + glycine plus pyridoxine (◑). Incubated in shaken culture at 37°.

Fig. 7. Growth of *Escherichia coli* B166 on lactate medium L in the presence (——) or absence (– – –) of glycolaldehyde, 5×10^{-4}M. Other supplements were: serine + glycine, 10^{-3}M (○); pyridoxine, 10^{-6}M (●); serine + glycine plus pyridoxine, (◑); serine + glycine plus activated glucose (□). Incubated in shaken culture at 37°.

Fig. 8. Growth of *Escherichia coli* B166 on lactate medium L containing glycolaldehyde (5×10^{-4}M) and inactive glucose (10 mg./ml.). Other supplements were: serine + glycine, 10^{-3}M (○); pyridoxine, 10^{-6}M (●); serine + glycine plus pyridoxine (◑). Incubated in shaken culture at 37°.

Apparent glycolaldehyde content of activated glucose. It was calculated from the results of Figs. 1 and 5 that activated glucose (10 mg./ml.) might contain from 1 to 4×10^{-4}M glycolaldehyde. A glucose solution of strength and pH value identical with that of medium G2 (10 mg./ml.; pH 7) was autoclaved under the standard conditions of 7 min. at 115° (10 lb./sq.in.) and the glycolaldehyde content of the product estimated chemically. About 2×10^{-4}M was found to be present (using an unautoclaved solution as blank). The dose-response curve with increasing concentrations of activated glucose could be superimposed upon the standard curve. Glycolaldehyde formation under other conditions of autoclaving was also determined (Fig. 3). Increasing ability to support growth on serine + glycine was matched by increasing glycolaldehyde content. Conditions which decreased biological activity (greater degree of heating or of alkalinity) still produced the maximum glycolaldehyde concentration; this may be a further indication that the diminished activity was due to concurrent formation of inhibitory substances.

Specificity of requirement for glycolaldehyde. A number of substances either related structurally to glycolaldehyde, or known to function under other circumstances as donors or acceptors of 'active glycolaldehyde' (Horecker & Mehler, 1955), did not replace glycolaldehyde in promoting growth of *Escherichia coli* B166 on serine + glycine. The compounds tested over a range of concentrations (10^{-6} to 2×10^{-3} M) in Seitz-filtered solution, included: ethanolamine, ethyleneglycol, glycollic, glyoxylic and oxalic acids, glycolaldehyde-2-phosphate, D-erythrose-4-phosphate, L-xylulose-5-phosphate, sedoheptulose-7-phosphate and L-erythrulose. It is possible that the phosphate esters do not permeate the organism.

Table 6. *Dihydroxyfumaric acid and β-hydroxypyruvic acid-supported growth of* Escherichia coli B166 *on medium* G2 *with serine + glycine*

All compounds were added aseptically as Seitz-filtered solutions. Basal medium was G2 with serine + glycine (10^{-3} M). Static cultures were incubated for 80 hr. at 37°.

	Growth (EEL reading) with		
Final conc. (M)	Glycol-aldehyde	Dihydroxy-fumaric acid	β-Hydroxy-pyruvic acid
4×10^{-3}	0	—	7
10^{-3}	14	12	10
5×10^{-4}	13·5	10	8·5
2×10^{-4}	11	5	5
5×10^{-5}	9	0	0
10^{-5}	7	0	0
10^{-6}	0	0	0

Dihydroxyfumaric acid and β-hydroxypyruvic acid were active (Table 6), but since the required concentration was about ten times that of glycolaldehyde this might have been due to spontaneous or metabolic conversion to glycolaldehyde. The spontaneous decomposition of dihydroxyfumaric acid is utilized for the chemical preparation of glycolaldehyde (Powers *et al.* 1955) and a solution of the acid kept at 60° for 30 min., assayed with organism B166 as if it had in fact been converted to its molar equivalent of glycolaldehyde. Difficulty was met in the colorimetric assay of glycolaldehyde in solutions of β-hydroxypyruvic acid, but maximum and minimum values for glycolaldehyde in a 5×10^{-3} M solution were 5×10^{-4} and 3×10^{-4} M respectively (see also Milhaud, Benson & Calvin, 1956).

Specificity of the amino acid requirement in the presence of glycolaldehyde. The response of strain B166 to amino acid mixtures was studied at various temperatures since mutant DW will grow at 25° in the absence of added vitamin B_6 when provided with a comprehensive mixture of amino acids (see below). When incubated at either 25 or 37°, growth of organism B166 took place on medium G2 supplemented with casein hydrolysate only when glycolaldehyde

(10^{-3}M) was also added. With a mixture of amino acids not including serine or glycine, growth in the presence of glycolaldehyde was less than with serine + glycine plus glycolaldehyde. The growth obtained in the absence of serine + glycine was due entirely to the DL-threonine content of the amino acid mixture (Table 7). Addition of the amino acid mixture without serine or glycine (AA-SG) decreased the pyridoxine requirement of the mutant only very slightly.

The quantitative requirements of organism B166 for serine + glycine were unchanged when 10^{-3}M-glycolaldehyde was used in place of activated glucose in medium G2 with or without pyridoxine.

Table 7. *Growth of* Escherichia coli B166 *in the presence of amino acids*

Supplements: glycolaldehyde, 10^{-3}M; pyridoxine, 10^{-6}M; serine + glycine, 10^{-3}M; DL-threonine, 10^{-3}M. AA-SG; amino acid mixture (each at 5×10^{-3}M) not containing serine + glycine used in 1/10 dilution. AA + PP + V; amino acids plus purines, pyrimidines and vitamins used in 1/10 dilution. Basal medium was G2 and incubation was in static culture for 84 hr. at 37°.

	Growth (EEL reading)
Glycolaldehyde	0
+ serine + glycine	12
+ AA-SG	7
+ DL-threonine	8
Pyridoxine	21
+ serine + glycine	24
+ AA + PP + V	22

Effect of changed conditions of culture. Under the usual conditions of aerobic culture at 37° and pH 7 there was an evident difference between the response of the organism to vitamin B_6 and to glycolaldehyde plus serine + glycine (Fig. 6). Although anaerobic growth was overall less good than that obtained aerobically the response of strain B166 to the compounds of interest remained relatively unchanged. The superiority of 10^{-3}M-glycolaldehyde to activated glucose in medium G2 was a little more marked (Table 8).

At 25°, aerobic growth in the presence of added pyridoxine alone was much delayed (110 hr.), but the decrease in incubation temperature had only a slight effect upon growth in the absence of added vitamin B_6 (Table 8). Growth on pyridoxine was again more adversely affected than that obtained on serine + glycine plus glycolaldehyde when the pH value of the medium was decreased to 6·5 (Table 8).

Escherichia coli B166 was most sensitive to inhibition by 4-deoxypyridoxine when growing in the presence of pyridoxine. Addition of serine + glycine decreased the sensitivity, while growth in the absence of vitamin B_6 was least affected. The organism was also most susceptible to inhibition by isoniazid when growing on vitamin B_6. The addition of serine + glycine to the pyridoxine-containing medium decreased sensitivity to the greatest extent. Growth on serine + glycine plus glycolaldehyde showed an intermediate degree of resistance.

Table 8. *Effect of changes in cultural conditions on growth of* Escherichia coli B166 *in the presence and absence of pyridoxine*

Basal medium G2 with supplements as shown of DL-serine + glycine, 10^{-3} M; activated glucose, 10 mg./ml.; glycolaldehyde, 10^{-3} M; pyridoxine, 10^{-6} M. Incubation was for 80 hr. at the temperature and under the conditions shown. Aerobic cultivation in sloped tubes in air. Anaerobic cultivation in upright tubes in modified Kilner jar containing 5% (v/v) CO_2 in N_2.

	Growth (EEL readings)			
Atmosphere	Anaerobic	Aerobic		
Temperature	37°	37°	25°	37°
pH	7·0	7·0	7·0	6·5
Supplement				
Nil	0	0	0	0
Pyridoxine	8	13	0*	8·5
Serine + glycine +				
activated glucose	2·5	10·5	—	—
glycolaldehyde	4·5	11	9	10·5
pyridoxine	11	20	15	15

* Some growth on prolonged incubation.

Characteristics of other vitamin B_6 *auxotrophs of* Escherichia coli

Mutants showing an effect with serine + glycine. Strain B167 closely resembled B166 under all conditions tested, i.e. in its ability to grow in the absence of added vitamin B_6 only when both serine + glycine and glycolaldehyde were provided, and in the enhancement and acceleration of its growth on pyridoxine by serine + glycine. There was no growth on media G2 or L with serine + glycine or glycolaldehyde as sole supplements.

Strain 22-29 differed from the other two members of this group in being able to grow on serine + glycine alone, and eventually, though only slightly, when glycolaldehyde was the sole supplement to medium G2. Growth on pyridoxine alone was again belated and suboptimal, but best growth occurred with a mixture of serine + glycine plus either pyridoxine or glycolaldehyde (Fig. 9).

Mutants showing no serine + glycine effect. Vitamin B_6 was required by strain DW for growth at 37°. At this temperature, it grew well with only short delay on medium G2 supplemented with 10^{-6} M-pyridoxine. The final extent of growth was increased by the concurrent addition of a comprehensive amino acid mixture, but in this serine and glycine played no special role. Glycolaldehyde (10^{-3} M) alone, or in admixture with serine + glycine (10^{-3} M), was without effect.

At 25° growth was possible without added vitamin B_6 when an acid-hydrolysate of casein, or a comprehensive amino acid mixture was supplied (Wijesundera, 1954). Serine and glycine seemed to have no part in this replacement action. Fairly good growth was obtained in medium G2 supplemented with DL-alanine plus DL-methionine (5×10^{-4} M) (Table 9). Glycolaldehyde at concentrations below 10^{-3} M had no observable effect in the presence or absence of added vitamin B_6.

Strain M2 was isolated in the course of a specific search for pyridoxine-requiring mutants amongst the products of u.v. irradiation of wild type *Escherichia coli*. It showed an absolute requirement for vitamin B_6 over a temperature range of 25–37°, in media based either on glucose or lactate;

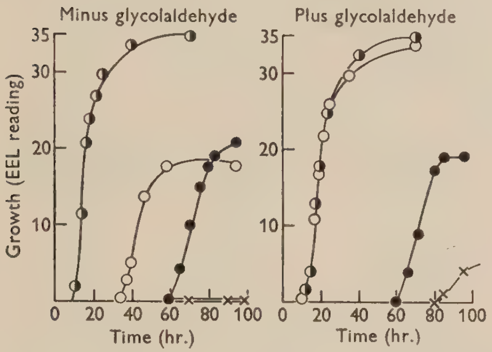

Fig. 9

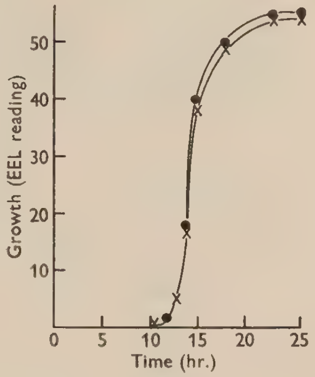

Fig. 10

Fig. 9. Growth of *Escherichia coli* 22-99 on medium G2 with and without glycolaldehyde (10^{-3}M). No further supplement (×); serine + glycine, 10^{-3}M (○); pyridoxine, 10^{-6}M (●); serine + glycine plus pyridoxine (◑). Incubated in shaken culture at 37°.

Fig. 10. Growth of auxotroph *Escherichia coli* M2 in medium G2 containing pyridoxine, 10^{-7}M (●), compared with growth of parent organism *Escherichia coli* strain 15 in unsupplemented medium G2 (×). Both incubated in shaken culture at 37°.

Table 9. *Growth of* Escherichia coli DW *at 25° on supplemented medium* G2

Supplements: pyridoxine, 10^{-6}M; amino acid mixtures used in 1/10 dilution to give final amino acid concs. of 5×10^{-4}M (each); DL-alanine, 10^{-3}M; DL-methionine, 5×10^{-4}M. Incubated in static culture 86 hr. at 25°.

Supplement	Growth (EEL reading)
Nil	0
Pyridoxine	17
Casein hydrolysate	16
Amino acid mixture AA	17
Amino acid mixture AA-SG	20
DL-Alanine + DL-methionine	15
DL-Alanine	< 1
DL-Methionine	0

growth comparable with that of the parent strain was obtained with 10^{-7}M-pyridoxine (Fig. 10). Occasionally, heavy growth appeared in a tube containing a casein hydrolysate supplement, but whenever this took place, examination proved that reversion had occurred. In the usual media the organism was stable even at very suboptimal concentrations of pyridoxine. Serine + glycine (10^{-3}M) had no effect either alone, or in admixture with 10^{-3}M-glycolaldehyde (Table 10).

When growth of strain M2 was limited by suboptimal concentrations of pyridoxine (4×10^{-8}M) the organisms clumped together producing an abnormal appearance in shaken culture; the culture medium was found to contain abnormally high concentrations of keto acids.

The results obtained with all the mutants used are summarized and compared in Table 11.

Table 10. *The pyridoxine requirement of* Escherichia coli M2

Basal medium G2 was supplemented as shown with serine + glycine, 10^{-3} M, and glycolaldehyde, 10^{-3} M. Incubated in static culture 42 hr. at 37°.

	Growth (EEL reading) with			
Pyridoxine conc. (M)	No addition	Serine + glycine	Glycol-aldehyde	Serine + glycine + glycolaldehyde
10^{-6}	27	27	27	27
10^{-7}	27	27	27	27
5×10^{-8}	22	22	8	< 1
2×10^{-8}	0	0	0	0

Table 11. *Summary of characteristics of vitamin* B_6 *mutants of* Escherichia coli

Growth response indicated by + + +, + +, ± +, etc., obtained in medium G2 supplemented as shown with serine + glycine 10^{-3} M; glycolaldehyde 10^{-3} M; pyridoxine 10^{-6} M.

	Supplement					
Mutant	None	Pyridoxine	Serine + glycine + pyridoxine	Serine + glycine + glycol-aldehyde	Serine + glycine	Glycol-aldehyde
B166	0	(+ +)	+ + +	± +	0	0
B167	0	(+ +)	+ + +	± +	0	0
22-99	0	(+ +)	+ + +	+ + +	(+ +)	(±)
DW	0	+ +	+ +	0	0	0
M2	0	+ + +	+ + +	0	0	0

( ) denotes appreciable delay before beginning of growth.

DISCUSSION

Glycolaldehyde and activated glucose. Glycolaldehyde was able to reproduce in every particular the biological activity of heated glucose solutions with *Escherichia coli* strain B166; an optimal concentration consistently improved upon their action. Even the observation that glycolaldehyde could at no concentration satisfactorily substitute for activated glucose in the lactate medium was fully explained by the concurrent action of unchanged glucose. The assay of the glycolaldehyde content of activated glucose samples by the Dische-Borenfreund method is sufficiently accurate for it to be concluded that (*a*) sufficient glycolaldehyde is present to account for the biological activity of the samples, and (*b*) the glycolaldehyde is formed only during the specific autoclaving procedure necessary for the production of activated glucose. Glycolaldehyde formation from glucose during alkaline autoclaving would probably proceed by way of scission of the 1:2-enediol intermediate into C_2

and C_4 fragments (Fig. 11). This accords with the finding that the sugar alcohols were not activated by autoclaving, for these compounds would be unable to form the enediolate ion. Again, scission at this position apparently requires migration of the hydrogen atom from the C-3 hydroxyl group; this could not occur in 3-O-methylglucose, which was found not to form the active factor.

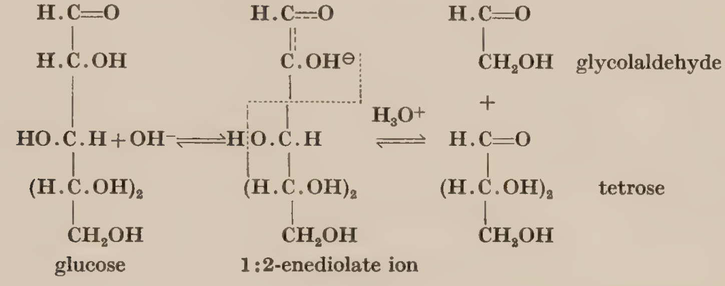

Fig. 11

Several instances are known of the development of biological activity during heat sterilization of glucose-containing media (Orla-Jensen, 1933; Rose & Peterson, 1949; Cheldelin, 1954; Hachisuka, Kato, Asano & Kuno, 1955; Ramsey & Lankford, 1956; Field & Lichstein, 1957; Sergeant, Lankford & Traxler, 1957). When the medium contains amino acids, Maillard-type, or other, interactions between these and the hexoses have been described. For example, N-D-glucosylglycine has been found to possess the same growth stimulating activity for *Lactobacillus gayonii* as medium-autoclaved glucose (Rogers, King & Cheldelin, 1953). Scission products have occasionally been held responsible for the growth stimulatory or inhibitory effects of glucose autoclaved in the absence of amino compounds; pyruvate, for example, replaced autoclaved glucose for *Streptococcus salivarius* and *Lactobacillus bulgaricus* (Smiley, Niven & Sherman, 1943; Snell, Kitay & Hoff-Jørgensen, 1948). However, the present study appears to be the first to implicate glycolaldehyde as a component of alkaline autoclaved glucose of possible biological significance.

Specificity of requirement for free glycolaldehyde. It is noteworthy that of all the vitamin B_6-requiring mutants of *Escherichia coli* examined, only with those whose growth on pyridoxine was accelerated and enhanced by serine + glycine could any action of glycolaldehyde be demonstrated. Although ethanolamine has been suggested as a possible precursor of free glycolaldehyde in animal tissues (Weissbach & Sprinson, 1953) this compound could not substitute for glycolaldehyde with *E. coli* B166, nor would several reported products of glycolaldehyde metabolism, e.g. glycollic acid, glyoxylic acid, glycine (Kun, Dechary & Pitot, 1954; Friedmann, Levin & Weinhouse, 1956), ethylene glycol (Sable, 1952; Buyze, 1955), and erythrulose (Horecker & Mehler, 1955). The activated two carbon fragment transferred in transketolase-catalysed reactions has tentatively been identified with 'active glycolaldehyde' or glycolaldehyde-thiamine pyrophosphate (Horecker, Smyrniotis & Klenow, 1953). Whether free glycolaldehyde can enter into equilibrium with the 'active glycolaldehyde' fragment is not known. The isotopic data of Friedmann *et al.* (1956) suggest that in animal tissues it cannot. This would explain why in the present study none of the 'active glycolaldehyde' donors tested was capable of substituting for free glycolaldehyde (though it is further possible that the phosphate esters were not available to the intact organism).

The high optimal concentration of glycolaldehyde (5×10^{-4} to 10^{-3}M) suggests that if its sole action is in the replacement of vitamin B_6 it is either very rapidly destroyed by the organism or acts indirectly. Free glycolaldehyde is in fact metabolized extensively and rapidly by growing cultures and washed suspensions of *Escherichia coli* B166 (Mr C. Mulder, personal communication). In contrast to serine + glycine, glycolaldehyde seems to have no specific action on the organism other than in its role in vitamin B_6 replacement; under no circumstances did glycolaldehyde exercise any effect in the presence of pyridoxine.

The vitamin B_6 *auxotrophs of* Escherichia coli. These are divisible into two groups differentiated by their response to serine + glycine and glycolaldehyde (Table 11). It is difficult to believe that those strains (B166, B167 and 22-99) for which these compounds can substitute for vitamin B_6 are true vitamin B_6 auxotrophs, for no concentration of any form of vitamin B_6 is able to evoke maximal growth in the absence of serine + glycine. The amino acids seemingly serve a double function, for besides their growth-enhancing effect in the presence of pyridoxine they must be concerned with glycolaldehyde in replacing vitamin B_6; if this is not so glycolaldehyde alone would be expected to evoke at least the suboptimal growth given by pyridoxine. The organisms are thus subject to a multiple metabolic block and must be at least considered as serine + glycine plus vitamin B_6 auxotrophs. A further complication is introduced by the finding that the magnitude of the growth response of strain B166 to serine + glycine plus glycolaldehyde was dependent upon the basal medium used, and that (unlike 22-99) maximal growth is never obtained in the absence of added vitamin B_6. A further substance may be concerned in the vitamin B_6-replacement action which is more readily formed from glucose than lactate. Could this be provided preformed, growth on serine + glycine plus glycolaldehyde might at least attain that degree found with serine + glycine plus pyridoxine. If the same substance had still further functions in these mutants, their growth in its presence together with serine + glycine and either pyridoxine or glycolaldehyde might reach the maximum attained by the prototroph. A fuller appraisal of the growth characteristics of these mutants has been attempted elsewhere (Morris, 1958).

It is considered that *Escherichia coli* M2 alone of all the mutants studied is a true vitamin B_6 auxotroph in that its requirement for the vitamin is absolute and its growth response to pyridoxine maximal. The ability of strain DW to grow in the absence of added vitamin B_6 at 25° is probably due to its metabolic block being incomplete at this temperature.

It has not been possible from the growth properties to establish with certainty the nature of the biochemical lesion or lesions in the mutants studied. In particular, it cannot be decided whether serine + glycine and glycolaldehyde decrease the need for vitamin B_6 or take part in its synthesis. An attempt to discriminate between these two possibilities by a direct study of vitamin B_6 synthesis in the light of the present results will be described in the following paper (Morris, 1959).

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REFERENCES

ABELSON, P. H. (1954). Amino acid biosynthesis in *Escherichia coli*: isotopic competition with C^{14}-glucose. *J. biol. Chem.* **206**, 335.

ADELBERG, E. A. & MYERS, J. W. (1953). Modification of the penicillin technique for the selection of auxotrophic bacteria. *J. Bact.* **65**, 348.

BALLOU, C. E., FISCHER, H. O. L. & McDONALD, D. L. (1955). The synthesis and properties of D-erythrose-4-phosphate. *J. Amer. chem. Soc.* **77**, 2658.

BUYZE, G. (1955). *De koolhydraatstofwisseling van* Lactobacillus brevis. (English summary.) Thesis: University of Utrecht.

CHELDELIN, V. H. (1954). New and unidentified growth factors. In *The Vitamins*, **3**, 589. Ed. Sebrell, W. H. & Harris, R. S. New York: Academic Press Inc.

CUTINELLI, C., EHRENSVÄRD, G., REIO, L., SALUSTE, E. & STJERNHOLM, R. (1951). Acetic acid metabolism in *Escherichia coli*. I. General features and the metabolic connexion between acetate and glutamic acid, aspartic acid, glycine, alanine, valine, serine and threonine. *Acta chem. scand.* **5**, 353.

DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. *J. Bact.* **60**, 17.

DENMAN, R. F., HOARE, D. S. & WORK, E. (1955). Diaminopimelic acid decarboxylase in pyridoxine-deficient *Escherichia coli*. *Biochim. biophys. Acta*, **16**, 442.

DISCHE, Z. & BORENFREUND, E. (1949). A specific color reaction of glycolic aldehyde. *J. biol. Chem.* **180**, 1297.

EULER, H. VON & MARTIUS, C. (1933). Über Redukton (Enol-Tartronaldehyd) und Ascorbinsäure. *Liebigs Ann.* **505**, 73.

EVANS, W. L. (1942). Some less familiar aspects of carbohydrate chemistry. *Chem. Rev.* **31**, 537.

FIELD, M. F. & LICHSTEIN, H. C. (1957). Factors affecting the growth of *Propionibacteria*. *J. Bact.* **73**, 96.

FRIEDEMANN, T. E. (1927). The action of alkali and hydrogen peroxide on glyoxals. *J. biol. Chem.* **73**, 331.

FRIEDMANN, B., LEVIN, H. W. & WEINHOUSE, S. (1956). Metabolism of glycolaldehyde in the rat. *J. biol. Chem.* **221**, 665.

GIBSON, F. W. E. (1952). *The Metabolism of Methionine by Micro-Organisms*. D.Phil. Thesis: University of Oxford.

HACHISUKA, Y., KATO, N., ASANO, N. & KUNO, T. (1955). Studies on spore germination. II. Effect of caramels from sugars and other carbon sources on spore germination. *J. Bact.* **69**, 407.

HEYNINGEN, W. E. VAN & GLADSTONE, G. P. (1953). The neurotoxin of *Shigella shigae*. III. The effect of iron on production of the toxin. *Brit. J. exp. Path.* **34**, 221.

HORECKER, B. L. & MEHLER, A. H. (1955). Carbohydrate metabolism. *Annu. Rev. Biochem.* **24**, 207.

HORECKER, B. L., SMYRNIOTIS, P. Z. & KLENOW, H. (1953). The formation of sedoheptulose phosphate from pentose phosphate. *J. biol. Chem.* **205**, 661.

Kun, E., Dechary, J. M. & Pitot, H. C. (1954). The oxidation of glycolic acid by a liver enzyme. *J. biol. Chem.* **210**, 269.

Meinhart, J. O. & Simmonds, S. (1955). Serine metabolism in a mutant strain of *Escherichia coli* strain K-12. *J. biol. Chem.* **213**, 329.

Milhaud, G., Benson, A. A. & Calvin, M. (1956). Metabolism of pyruvic acid-2-C^{14} and hydroxypyruvic acid-2-C^{14} in algae. *J. biol. Chem.* **218**, 599.

Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). Sur la biosynthese de la β-galactosidase (lactase) chez *Escherichia coli.* La spécificité de l'induction. *Biochim. biophys. Acta*, **7**, 585.

Morris, J. G. (1958). *The Synthesis of Vitamin* B_6 *and Related Substances by Micro-organisms.* D.Phil. Thesis: University of Oxford

Morris, J. G. (1959). The synthesis of vitamin B by some mutant strains of *Escherichia coli. J. gen. Microbiol.* **20**, 597.

Müller, H., Montigel, C. & Reichstein, T. (1937). Reine *l*-Erythrulose (*l*-2-keto-tetrose). *Helv. chim. Acta*, **20**, 1468.

Orla-Jensen, A. D. (1933). Hitherto unknown activators for the growth of lactic acid bacteria. *J. Soc. chem. Ind., Lond.*, **52**, 374.

Pigmann, W. W. & Goepp, R. M. (1948). *Chemistry of the Carbohydrates.* New York: Academic Press Inc.

Powers, H. H., Tabakoglu, G. & Sable, H. Z. (1955). Glycolaldehyde. *Biochem. Prep.* **4**, 56.

Ramsey, H. H. & Lankford, C. E. (1956). Stimulation of growth initiation by heat degradation products of glucose. *J. Bact.* **72**, 511.

Rogers, D., King, T. E. & Cheldelin, V. H. (1953). Growth stimulation of *Lactobacillus gayoni* by *N*-D-glucosylglycine. *Proc. Soc. exp. Biol., N.Y.* **82**, 140.

Rose, D. & Peterson, R. (1949). Influence of the amino acid-dextrose reaction on growth of lactic acid bacteria. *Canad. J. Res.* **27** B, 428.

Sable, H. Z. (1952). Pentose metabolism in extracts of yeast and mammalian tissues. *Biochim. biophys. Acta*, **8**, 687.

Sergeant, T. P., Lankford, C. E. & Traxler, R. W. (1957). Initiation of growth of *Bacillus* species in a chemically defined medium. *J. Bact.* **74**, 728.

Smiley, K. L., Niven, C. F. & Sherman, J. M. (1943) The nutrition of *Streptococcus salivarius. J. Bact.* **45**, 445.

Snell, E. E., Kitay, E. & Hoff-Jørgensen, E. (1948). Carbohydrate utilisation by a strain of *Lactobacillus bulgaricus. Arch. Biochem.* **18**, 495.

Sprinson, D. B. & Chargaff, E. (1946). A study of β-hydroxy-α-keto acids. *J. biol. Chem.* **164**, 417.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (945). *Manometric Techniques and Tissue Metabolism*, 1st ed. Minneapolis: Burgess Publ. Co.

Weissbach, A. & Sprinson, D. B. (1953). The metabolism of 2-carbon compounds related to glycine. I. Glyoxylic acid. II. Ethanolamine. *J. biol. Chem.* **203**, 1023, 1031.

Wijesundera, S. (1954). *Methionine and Related Compounds in the Metabolism of Micro-Organisms.* D.Phil. Thesis: University of Oxford.

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Morris, J. G. (1959). *J. gen. Microbiol.* **20**, 597–604

The Synthesis of Vitamin B_6 by some Mutant Strains of *Escherichia coli*

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SUMMARY: *Escherichia coli* B166 synthesizes as much vitamin B_6 when growing on serine or glycine plus glycolaldehyde as does its parent (*E. coli* B) on unsupplemented medium. Suspensions of strain B166 harvested after growth on serine + glycine plus glycolaldehyde, and incubated with glucose and NH_4^+, only synthesize vitamin B_6 when glycolaldehyde is added; serine + glycine increases the synthesis. Strain 22-99 produces more vitamin B_6 when grown on glycolaldehyde plus serine + glycine than when it is grown on the amino acids alone, while, depending upon the conditions of growth, vitamin B_6 synthesis by suspensions is absolutely or partially dependent on serine + glycine plus glycolaldehyde. However, serine + glycine plus glycolaldehyde have little effect on the biosynthesis of vitamin B_6 by wild-type *E. coli* during growth, or in washed suspensions.

The growth of certain mutants of *Escherichia coli* (strains B166 and 22-99), which respond suboptimally to vitamin B_6, is more rapid and extensive when serine or glycine is also provided. The same organisms grow in the absence of added vitamin when supplied with either glycine or serine plus glycolaldehyde (Morris & Woods, 1959).

Serine or glycine plus glycolaldehyde would be able to replace vitamin B_6 for *Escherichia coli* B166 if they were the sole products of function of the vitamin when growth was taking place in an ammonium + glucose medium. This is most unlikely; an alternative explanation is that the mutant is able to synthesize an amount of the vitamin which is sufficient to satisfy all normal functions except those of synthesizing serine, glycine and glycolaldehyde. A third possibility is that glycolaldehyde and serine or glycine are concerned in the biosynthesis of vitamin B_6 by *E. coli*; the present work provides some evidence that this may be true for the mutants *E. coli* B166 and 22-99.

METHODS

Organisms. Escherichia coli PA15, a mutant strain requiring L-serine or glycine, was obtained from Dr Barbara Wright. The other *E. coli* auxotrophs were those used by Morris & Woods (1959).

Growth. The media, conditions of growth, and the method of assessment of growth were as described previously (Morris & Woods, 1959).

Suspensions. Organisms were harvested after maximal growth on chemically-defined media and washed twice with 0·1M-phosphate buffer containing 4×10^{-4}M-$MgSO_4$ before final suspension in the same buffer containing the test substrate. When it was desired to deplete the organisms of endogenous substrates a suspension of organisms (equiv. to 5 mg. dry wt./ml.) in 50 ml. of the above buffer was rocked in a large ⊥-tube at 37° for 2–3 hr. before re-harvesting and washing. Test incubations were with rocking for not more than 8 hr. at 37° in ⊥-tubes of 19 mm. diameter holding 10 ml. of suspension.

Extraction and assay of vitamin B_6. This was carried out by the method described by Morris, Hughes & Mulder (1959), using *Saccharomyces carlsbergensis* 4228*c*.

Miscellaneous. DL-Serine and glycine were frequently used in equimolar mixture referred to as serine + glycine, the molar concentration being given in terms of the total amino nitrogen. 'Activated glucose' refers to solutions of glucose in 0·04 M-phosphate buffer (pH 7·4) autoclaved at 115° for 7 min. All other chemicals were as described by Morris & Woods (1959).

RESULTS

The total amount of vitamin B_6 synthesized by wild-type *Escherichia coli* during growth on a simple glucose + ammonium + salts medium (G 2) increased commensurately with growth and did not significantly decrease thereafter, though the distribution of vitamin between organism and culture medium changed. The proportion of vitamin present in the organisms was high during the logarithmic phase of growth and then decreased rapidly at the onset of the stationary phase (Fig. 1). The decrease in internal vitamin B_6 after growth ceased could be accounted for by its increase in the culture medium. In a comparison of vitamin B_6 synthetic abilities, it is therefore insufficient to assay only the bacterial content of vitamin, for this will vary with the age of the culture.

The synthesis of vitamin B_6 *by mutant strains of* Escherichia coli *which respond to vitamin* B_6 *or to serine + glycine plus glycolaldehyde*

Synthesis of vitamin B_6 *during growth.* Production of vitamin B_6 by mutant B166 growing on medium G 2 supplemented with serine + glycine plus glycolaldehyde or activated glucose, was quantitatively similar to that of its parent strain (*Escherichia coli* B) growing on the unsupplemented basal medium. The results given (Table 1*a*) are in terms of the total vitamin B_6 content of the culture (organisms plus medium) at full growth. There was no increase in vitamin B_6 when the media were supplemented with casein hydrolysate, a mixture of purines and pyrimidines, and vitamins other than B_6.

Escherichia coli 22-99 grows optimally on serine + glycine plus glycolaldehyde and less well on serine + glycine alone (Morris & Woods, 1959). More vitamin B_6 was synthesized when the organism was grown on glycolaldehyde plus serine + glycine than when it was grown on the amino acids alone (Table 2*a*).

Synthesis of vitamin B_6 *by suspensions.* After growth of *Escherichia coli* B166 in the presence of 10^{-6} M-pyridoxine only about half of this compound was recoverable as assayable vitamin B_6; there was, however, complete recovery when a washed suspension was incubated aerobically with the same concentration of pyridoxine for 8 hr. at 37°. It was therefore possible to study vitamin B_6 synthesis by suspensions of this mutant.

Suspensions of strain B166 harvested from growth media containing pyridoxine, synthesized little vitamin B_6 when subsequently incubated in phosphate buffer containing Mg^{++}, NH_4^+, glucose, serine + glycine and glycolaldehyde. Organisms grown in the absence of added vitamin B_6 were able to synthesize the vitamin in much better yield. The magnitude of the synthesis differed from experiment to experiment even though great care was taken to grow, harvest and treat each batch of organisms identically. When such washed suspensions were supplied with glucose plus NH_4^+ some synthesis of vitamin B_6 always occurred, but this was invariably increased by the addition of glycolaldehyde. With serine + glycine and glycolaldehyde as sole substrates, some vitamin B_6 was formed, though not as much as when glucose was also present. The basal synthesis from glucose plus NH_4^+ might be due to the carry-over of some precursor(s) formed by the organisms during their growth on serine + glycine plus glycolaldehyde—the substrates whose effect on vitamin B_6 biosynthesis was to be tested. In further experiments, suspensions were incubated in buffer alone before use, in the hope of removing such endogenous material. Organisms so depleted were unable to synthesize vitamin B_6 from glucose plus NH_4^+ alone, but did so when glycolaldehyde (10^{-3} M) was added. Serine + glycine increased the synthesis but NH_4^+ was still required for

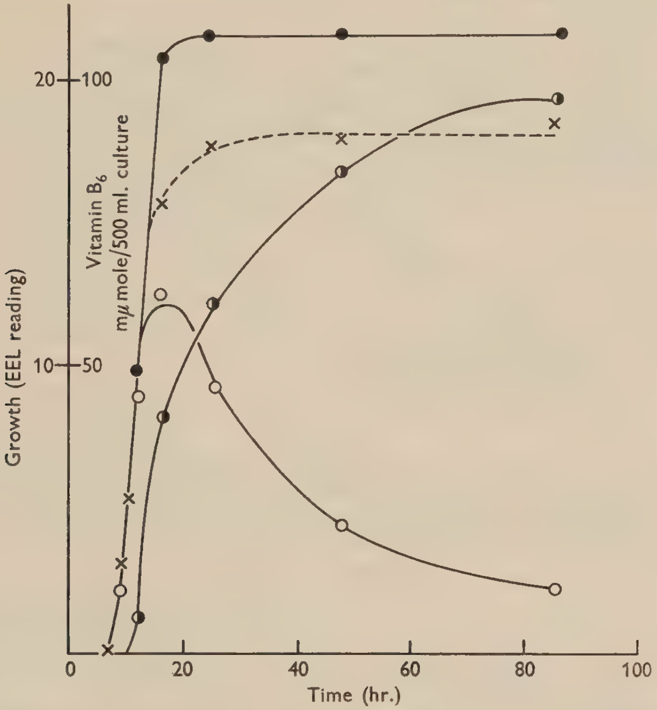

Fig. 1. Distribution of synthesized vitamin B_6 between organisms and culture medium during growth of *Escherichia coli* strain 15 on medium G2. Growth of organisms (× --- ×), vitamin B_6 content of organisms (○) and of culture medium (◑) giving total synthesis (●).

maximum vitamin production (Table 1*b*). The yield of vitamin B_6 again differed from batch to batch of organisms though all showed an absolute requirement for glycolaldehyde for any synthesis of the vitamin. Little synthesis occurred in the absence of glucose, which may serve both as a source of energy and of another compound which would otherwise have to be provided by endogenous metabolism.

Table 1. *Synthesis of vitamin* B_6 *by* Escherichia coli B166

(*a*) *During growth.* Medium G2 supplemented as shown with serine + glycine 10^{-3} M; glycolaldehyde 10^{-3} M; activated glucose 10 mg./ml. Cultures (500 ml.) were incubated aerobically for 40 hr. at 37°.

	Vitamin B_6 synthesis (mμmole pyridoxine/g. dry wt.)	
Supplements	Prototroph (strain B)	Auxotroph (strain B166)
None	465	No growth
Serine + glycine plus		
activated glucose	400	255
glycolaldehyde	470	420

(*b*) *By washed suspensions.* Depleted, washed organisms (equiv. 6 mg. dry wt./ml.) grown on medium G2 with serine + glycine and glycolaldehyde (10^{-3} M each) were suspended in phosphate buffer (0·1 M; pH 7·0), supplemented as shown with glucose 1 % (w/v); NH_4^+ as $(NH_4)_2SO_4$ 5×10^{-2} M; serine + glycine 10^{-3} M; glycolaldehyde 10^{-3} M. Incubated for 6 hr. at 37°.

	Vitamin B_6 biosynthesis (mμmole pyridoxine/g. dry wt.) Experiment			
Supplements	(*a*)	(*b*)	(*c*)	(*d*)
None	0	0	0	0
Serine + glycine + glycolaldehyde	—	—	—	4
Glucose plus				
serine + glycine	20	0	0	0
glycolaldehyde	20	0	0	0
glycolaldehyde + serine + glycine	320	140	220	120
Glucose + NH_4^+	10	0	0	0
Glucose + NH_4^+ plus				
glycolaldehyde	—	100	180	140
glycolaldehyde + serine + glycine	—	—	370	210

Since good growth of strain 22–99 took place on medium G2 with serine + glycine alone, it was considered possible that suspensions of the organism so grown might not show an obligatory requirement for glycolaldehyde for vitamin B_6 synthesis. Such suspensions proved capable of synthesizing vitamin B_6 to some extent from glucose plus NH_4^+. However, the synthesis was still further improved by the addition of glycolaldehyde. Suspensions of depleted organisms grown on serine + glycine plus glycolaldehyde synthesized vitamin B_6 only when provided with serine + glycine plus glycolaldehyde (Table 2*b*).

Table 2. *Biosynthesis of vitamin* B_6 *by* Escherichia coli 22-99

(*a*) *During growth.* Supplements (10^{-3} M) to medium G2 were as shown and the cultures (50 ml.) were incubated aerobically (with rocking in large ⊥-tubes) at 37° until maximum growth was achieved.

Supplements	Growth (EEL reading)	Total vitamin B_6 synthesis (mμmole pyridoxine/g. dry wt.)
None	0	0
Serine + glycine	21	180
Serine + glycine + glycolaldehyde	28	424

(*b*) *By washed suspensions.* Depleted, washed organisms (equiv. 6 mg. dry wt./ml.) grown on medium G2 with *A*, serine + glycine; *B*, serine + glycine plus glycolaldehyde, suspended in phosphate buffer (0·1 M, pH 7) with supplements as shown in concn. given in Table 1. Incubated for 6 hr. at 37°.

	Vitamin B_6 synthesis (mμmole pyridoxine/g. dry wt.) in presence of			
	NH_4^+		Serine + glycine	
Other additions	*A*	*B*	*A*	*B*
Glycolaldehyde	0	0	0	70
Glucose	75	0	60	0
Glucose + glycolaldehyde	135	0	180	180

Table 3. *Vitamin* B_6 *synthesis by* Escherichia coli *DW during growth at* 25°

Medium G2 was supplemented as shown with alanine, methionine, serine + glycine and glycolaldehyde (10^{-3} M each); amino acid mixture AA-SG in 1/10 dilution to give final individual amino acid concn. of 5×10^{-4} M. Cultures (50 ml.) were incubated aerobically at 25° until best growth was achieved

Supplements	Growth (EEL reading)	Total vitamin B_6 synthesis (mμmole pyridoxine/g. dry wt.)
None	0	0
DL-alanine + DL-methionine	26	33
AA-SG	22	28
AA-SG + serine + glycine + glycolaldehyde	23	73

Vitamin B_6 *synthesis by a temperature-sensitive vitamin* B_6 *auxotroph*

Strain DW, although showing an absolute requirement for vitamin B_6 at 37°, may be grown at 25° in the presence of any mixture of amino acids containing DL-alanine and DL-methionine; the amount of vitamin B_6 then synthesized is less than one-tenth of that formed by prototrophic strains (Table 3). When grown on medium G2 supplemented with (*a*) an amino acid mixture not including serine or glycine (AA-SG), or (*b*) the same mixture with 10^{-3} M each of serine + glycine plus glycolaldehyde, only a little more vitamin B_6 was produced under the latter conditions (Table 3).

Vitamin B_6 *synthesis by wild-type* Escherichia coli

Glycolaldehyde and serine + glycine (both at 10^{-3} M) alone or together had little effect on the amount of vitamin synthesized during growth of *Escherichia coli* strains 15 and B either on glucose-containing medium (G 2) or on the lactate medium (L) (Table 4*a*). Although best synthesis was in fact obtained by growth in the presence of a mixture of all these substances, the increase is of doubtful significance.

Serine + glycine and glycolaldehyde (10^{-3} M, each) also did not increase the production of vitamin B_6 by washed suspensions of *Escherichia coli* B above that obtained from glucose plus NH_4^+ (Table 4*b*). Addition of serine + glycine plus glycolaldehyde to the original growth medium had no effect on subsequent vitamin synthesis by harvested organisms.

Table 4. *Biosynthesis of vitamin* B_6 *by wild-type strains of* Escherichia coli

(*a*) *During growth.* Cultures (50 ml.) were incubated aerobically at 37° until best growth was achieved.

	Total vitamin B_6 synthesis (mμmole pyridoxine/g. dry wt.)		
Organism	Strain 15		Strain B
Basal medium	G 2	L	L + glucose
Other additions			
None	454	450	450
Serine + glycine + glycolaldehyde	570	452	600

(*b*) *By washed suspensions of strain* B. Washed organisms (7·5 mg./ml.) grown on medium G 2, suspended in phosphate buffer (0·1 M, pH 7·0) supplemented with Mg^{++}, and other supplements as shown, glucose 1 % (w/v); NH_4^+ as $(NH_4)_2SO_4$ 5×10^{-2} M; serine + glycine 10^{-3} M; glycolaldehyde 10^{-3} M. Incubated for 8 hr. at 37°.

Supplements	Vitamin B_6 synthesis (mμmole/g. dry wt.)
None	0
Glucose + NH_4^+	340
+ serine + glycine + glycolaldehyde	340

Vitamin B_6 *synthesis of a serine + glycine mutant of* Escherichia coli

It is conceivable that no effect of serine + glycine was demonstrable with prototrophic strains because these organisms are fully capable of synthesizing these amino acids from glucose plus NH_4^+. *Escherichia coli* PA 15 was therefore studied as a representative mutant which is incapable of *de novo* synthesis of serine and glycine. When this organism was grown with suboptimal concentrations of glycine the amount of vitamin B_6 synthesized (expressed in terms of amount/g. dry wt. organism) was actually greater than during maximal growth in the presence of an excess of glycine, while vitamin B_6 synthesis from glucose plus NH_4^+ by washed suspensions of organisms so grown was not increased by an excess of glycine (Table 5).

Table 5. *Biosynthesis of vitamin* B_6 *by the serine-glycine mutant* Escherichia coli *PA* 15

(*a*) *During growth.* Medium G2 (50 ml.) supplemented with glycine as shown. Incubated aerobically with rocking in ⊥-tubes at 37° until best growth was achieved.

Glycine concn. (M)	Growth (EEL reading)	Total vitamin B_6 synthesis	
		Concn. (M)	(mμmole/g. dry wt.)
5×10^{-2}	66	10^{-6}	500
10^{-2}	60	$1{\cdot}1\times10^{-6}$	565
5×10^{-3}	35	$1{\cdot}1\times10^{-6}$	1100
2×10^{-3}	15·5	$5{\cdot}5\times10^{-7}$	1550
10^{-3}	6	$2{\cdot}8\times10^{-7}$	1870
5×10^{-4}	3	$1{\cdot}4\times10^{-7}$	1870

(*b*) *By washed suspensions.* Washed depleted organisms (equiv. 6 mg. dry wt./ml.) grown on medium G2 with 10^{-3} M-glycine (a growth-limiting concentration), suspended in phosphate buffer (0·1 M, pH 7·0) supplemented as shown with glucose 10^{-1} M; NH_4^+ as NH_4Cl 10^{-1} M glycine 10^{-2} M; glycolaldehyde 10^{-3} M. Incubated aerobically for 6 hr. at 37°.

Supplement	Vitamin B_6 synthesis (mμmole/g. dry wt.)
None	0
Glucose + NH_4^+	800
Glucose + NH_4^+ +	
glycine	720
glycolaldehyde	800
glycine + glycolaldehyde	960

DISCUSSION

Serine and glycine have some sparing action on the vitamin B_6 requirement of *Escherichia coli* B166 which is manifested in a somewhat decreased pyridoxine requirement and increased resistance to inhibition by 4-deoxypyridoxine or isoniazid in the presence of these amino acids (Morris & Woods, 1959). However, it does not appear that this can be the sole explanation of all their effects upon the organism. It is unlikely that the replacement of vitamin B_6 is due solely to the establishment of conditions in which there is a reduced requirement for the vitamin for the following reasons: (*a*) serine + glycine plus glycolaldehyde have no marked pyridoxine-sparing action with auxotrophs M2 and DW; (*b*) glycolaldehyde alone has no pyridoxine-sparing effect; (*c*) the amount of vitamin B_6 synthesized by strain B166 when growing on serine + glycine plus glycolaldehyde equals that produced by the parent strain in unsupplemented medium. The last finding immediately suggests that serine + glycine and glycolaldehyde may be concerned with the actual synthesis of vitamin B_6 by this mutant.

It is probable that the growth of mutant DW at 25°, when vitamin B_6 synthesis is less than one-tenth that of wild-type strains, is a consequence of the metabolic block being incomplete at this temperature. In the presence of certain essential products of its function (in this instance, amino acids, particularly D-alanine and L-methionine) the decreased amount of vitamin that is now synthesized is sufficient to fulfil all other growth requirements.

That the situation with strains B166 and 22-99 is different from that in strain DW is confirmed by the results obtained with washed suspensions. These support the view that glycolaldehyde (and possibly serine + glycine as well) is concerned with the actual synthesis of the vitamin by the first two strains. However, it cannot be concluded that even glycolaldehyde is an actual reactant in the synthesis of vitamin B_6 by these mutants; it may act indirectly either by sparing a normal intermediate or by inhibiting a deleterious reaction. The role of the serine or glycine is confused by their ability also to accelerate and enhance growth of strains B166 and 22-99 in the presence of added pyridoxine (Morris & Woods, 1959), an action which cannot be explained by their possible participation in a sparing or synthesis of vitamin B_6. This stimulation of growth might be due to conversion of the vitamin into a more active form, or to increased permeability of the organisms to added vitamin. Yet maximal growth in the absence of glycine or serine was not obtained even in the presence of very high concentrations of all known forms of the vitamin. It is possible that interference with vitamin B_6 biosynthesis in the organism may be only one consequence of the presence of an incomplete metabolic block which also controls synthesis of serine and glycine.

No confirmation of a function of serine, glycine and glycolaldehyde in vitamin B_6 biosynthesis has been possible with prototrophic strains. Some restriction of vitamin B_6 biosynthesis must operate even in the wild-type organism and it is not inconceivable that the amount of synthesis normally attained *de novo* from glucose plus NH_4^+ cannot be surpassed when precursors are provided which, even if directly on the route of biosynthesis, are rather remote from the final product.

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REFERENCES

Morris, J. G., Hughes, D. T. D. & Mulder, C. (1959). Observations on the assay of vitamin B_6 with *Saccharomyces carlsbergensis* 4228. *J. gen. Microbiol.* **20**, 566.

Morris, J. G. & Woods, D. D. (1959). Inter-relationships of serine, glycine and vitamin B_6 in the growth of mutants of *Escherichia coli*. *J. gen. Microbiol.* **20**, 576.

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Effects of Extracts of Blue-Green Algae on Pigment Production by *Serratia marcescens*

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SUMMARY: Two extract preparations from each of twelve species of blue-green algae were tested for possible anti-pigmentation activities when applied to unpigmented cultures of *Serratia marcescens*. Extracts from eight of these species inhibited pigment formation, two of them completely. The two extraction methods gave similar results and there appears to be no difference in the effects produced by extracts from pure and impure algal cultures. Living or preserved samples of the algae containing the anti-pigmentation factor (APF) were also toxic to mice when whole or homogenized organisms were injected intraperitoneally. Numerous tests showed that the bacterial associates of the algae did not enter into the production of the APF or the toxic substance. The most effective extract concentration was 0·2 ml. extract/ml. broth, and within the range of concentrations employed, the degree of pigment inhibition was inversely proportional to the extract concentration. The possibility of using the anti-pigmentation test to determine the presence of toxic substances in blue-green algae is suggested.

Although the toxic effects of certain blue-green algae on wild and domestic animals are well known, little information is available about the antibacterial activities of these organisms or their exudates. It has been reported that *Microcystis aeruginosa* Kütz. possesses some antibacterial properties against certain Gram-positive and Gram-negative bacteria (Lefevre, Jakob & Nesbet, 1952; Wurtz, 1949), and that *Oscillatoria splendida* Grew, under certain conditions, secretes a volatile substance which kills bacteria and other microorganisms (Goryunova, 1955). Results obtained in this laboratory during the last three years confirm the belief that other Cyanophycean species, especially those which are toxic to higher animals, also possess antibacterial properties. During the course of this investigation on the effects of extracts of blue-green algae on certain forms of Enterobacteriaceae, the possible existence of an anti-pigmentation factor (APF) in certain of these extracts was indicated. Extracts which inhibited pigment formation by certain chromogenic bacteria did not affect their growth processes; and within the range of concentrations used the degree of pigment inhibition appeared to be inversely proportional to the concentration of the applied extract. The first indication of an anti-pigmentation activity was observed in cultures of *Serratia marcescens* Bizio. which had been treated with aqueous extracts from *Microcystis aeruginosa* Kütz. or from *Nostoc rivulare* Kütz. Further observations revealed that corresponding concentrations of extracts from certain other blue-green algae exhibited a similar but less pronounced inhibitory action. In view of these preliminary results, a survey was undertaken to determine the occurrence of the APF in a selected group of blue-green algae, including some of those which have been implicated in the death of higher animals, and to determine the concentrations of each extract required for the inhibition of pigment formation by *S. marcescens* Bizio.

It has been well established that the biosynthesis of prodigiosin, the tripyrrylmethane pigment of *Serratia marcescens* Bizio (Hubbard & Rimington, 1950) is influenced by such factors as radiation, temperature, pH value of the growth medium, age of the culture, chlorophyll, numerous organic substances, and by certain antibiotics (Kreitlow, 1942; Novelli, 1953; Weinberg, 1951; Weil, 1952; Bunting, 1950; Crichton & Lazarus, 1948; Williams, Green & Rappoport, 1956). In the present study, the factors temperature, light, pH value and age of cultures were controlled. Weil (1952) demonstrated that Terramycin and Aureomycin are capable of inhibiting the formation of prodigiosin by *S. marcescens*. Although the active principle considered in the present report has not been identified, preliminary studies indicate that its pigment-inhibition properties resemble those of Terramycin and Aureomycin.

METHODS

Blue-green algae tested. Nine unialgal, bacteria-contaminated cultures were obtained from the Indiana University stock culture collection for use in this study. The strain number is given in parentheses after each species: *Tolypothrix distorta* var. *symplocides* Hansg. (B424); *Anabaena catenula* (Kütz. Born. et Flah. (B375)); *A. variabilis* Kütz. (B377); *Nostoc ellipsosporum* (Desmaz.) Rabenh. (B383); *N. calcicola* Breb. (B382); *N. punctiforme* (Kütz). Hariot. (B384); *Calothrix membranacea* Schmidle (B379); *Oscillatoria tenuis* Ag. (B428); *Lyngbya* sp. (487). Pure cultures of *Microcystis aeruginosa* Kütz. were obtained from Dr G. C. Gerloff (University of Wisconsin) and *O. formosa* Bory (strain LB390) from the Indiana University stock culture collection. Samples of *N. rivulare* Kütz. and *M. aeruginosa* Kütz. were collected locally and the identifications were verified by Dr F. Drouet (Cryptogamic Herbarium, Chicago Natural History Museum). These two species were established in unialgal cultures but were not bacteria-free.

Jelly-like masses of *Nostoc rivulare* Kütz. were transferred to the laboratory where they were spread into thin layers on plastic sheets. The material was fan-dried at 30° for 24 hr., powdered in a Waring blendor and stored in sealed containers. The toxicity of the algae at different stages of preservation was tested by intraperitoneal injections in mice.

Algae grown in the laboratory were dried under reduced pressure at 60° and were stored in separate containers.

Culturing the algae. In order to provide an adequate and reliable supply of algal material, cultures of the species employed were established in carboys or in Erlenmeyer flasks. The carboys were aerated with compressed air and the flasks by agitation on a mechanical rocking device. Constant illumination (500 ft.-c.) was provided by 40 W. cool-white fluorescent tubes, and the room temperature was thermostatically controlled at 26–30°. Several growth media were tested. The most effective were: Gerloff, Fitzgerald & Skoog (1952) modification of no. 10 medium of Chu (1942); Benecke medium (De, 1939); Burkholder (1955) medium containing 20 ml. soil extract (Pringsheim, 1949), 0·2 g. KNO_3, 0·002 g. K_2HPO_4, 0·002 g. $MgSO_4.7H_2O$, 100 ml. distilled water. Stock cultures were maintained on Cyanophycean agar or soil extract agar (Starr, 1956) in a stock culture room provided with a constant temperature of 20–24°, light intensity of 50–75 ft.-c., and relative humidity of 50–60%.

Preparation of extracts. Several techniques were tested in an effort to obtain extracts which contained the active APF principle. Of these an adsorption and elution technique was inferior to the other extraction procedures; attempts to elute completely APF which had been adsorbed on activated charcoal gave unsatisfactory results.

Thirteen organic solvents were tested in the preparation of crude extracts: acetone, chloroform, carbon tetrachloride, ethyl acetate, benzene, light petroleum (boiling range 35·7–36·7°), toluene, pentane, methanol pyridine, ethylene dichloride, methylethyl ketone, *n*-hexane, and ethanol. These solvents were used successively on the same sample of dried algae for selective extraction, and separately on different samples. In each case ethanol was found to be the most effective solvent for extracting APF. In the preparation of the extracts by this method, 50 g. preserved algae was ground in a ball mill and thoroughly mixed with three times its volume of purified sand. Ethanol (500 ml.) was percolated through the mixture at room temperature and evaporated under reduced pressure at 60°. The residue was viscous and tacky and approached a brittle solid when thoroughly dried. This residue was agitated with sterile double-distilled water for 2 hr. and after the suspended particles had settled, the aqueous extract was removed and tested on nutrient broth and nutrient agar for the presence of bacteria.

The most satisfactory results were obtained by the use of distilled water as the solvent for the APF. A sample (0·5 g.) of algae was ground in a ball mill for 12 hr. Sterile distilled water (100 ml.) was added and ball-milling continued for 1 hr. Microscopic examination of the suspension revealed that after the ball-milling the algal cells were thoroughly disintegrated. The mixture was centrifuged and the supernatant fluid removed for filtration. In order to provide adequate sterilization of the filtrate and to avoid the possible adsorption of APF by paper or porcelain filters, a sintered-glass bacterial filter was used. Samples of the aqueous filtrate were tested for sterility on nutrient agar and in nutrient broth. The nature and amount of APF in the algae are unknown. Hence, its concentration can be expressed only in reference to weight of plant material to the volume of solvent employed, i.e. 0·5 g. dried algae/ 100 ml. distilled water. In case the APF was lost on storage in an aqueous solution, only fresh extracts were tested.

From each of the species of algae mentioned above two kinds of extract were prepared, i.e. by organic solvent and by distilled water extraction. The pH value of the prepared extracts was 7·5–7·7.

Testing of extracts. The test organism used was *Serratia marcescens*, strain 261 (Indiana University stock culture collection). This strain was selected because of its ability to produce intense pigmentation in Difco nutrient broth, the medium used throughout this study. In nutrient broth, maximum growth and pigmentation occurred within 72 hr. when the cultures were incubated at 20–25° in total darkness.

To three tubes each containing 48 mg. dehydrated nutrient broth, were added 5, 4 or 3 ml. of distilled water. After the tubes were autoclaved at 121° for 20 min. and cooled, 1, 2 and 3 ml. sterile aqueous filtrate respectively were added to the tubes, so that the nutrient broth concentration and the volume of medium in the tubes was the same throughout. The final concentration of extract in the three tubes was 0·2, 0·5 and 1 ml./ml. medium. A similar tube of medium without extract was also prepared. Each of the four tubes was inoculated with a loopful of a 24 hr. culture of *Serratia marcescens*. Two uninoculated control tubes were also used, one containing medium only and the other only sterile aqueous filtrate plus water to 6 ml.

A second series of six tubes was prepared as above to test the anti-pigmentation activity of the aqueous extract. The final pH value of the experimental and control cultures remained at 7·5–7·7. The twelve tubes in the two series were mechanically rotated for 72 hr. in a dark room at 26–30°.

Bacterial associates of each species of algae employed were isolated and tested for possible anti-pigmentation properties. Cultures (48 hr.) of these bacteria were centrifuged for 1 hr., the deposit was removed, the organisms washed in two changes of normal saline and then suspended in 2 ml. normal saline. These bacterial suspensions were applied to cultures of *Serratia marcescens* in concentrations corresponding to those of the algal extracts. Control cultures were prepared as in the preceding tests. The tubes were mechanically rotated under environmental conditions identical with those used in the algal extract experiments.

Extraction of prodigiosin. For this procedure, Hubbard & Rimington (1950) method was modified. Water lost from the culture through evaporation during the 72 hr. incubation period was restored. Acetone containing 2% (v/v) of glacial acetic acid (6 ml.) was mechanically shaken with the culture for 30–45 min. The prodigiosin was then extracted three times with light petroleum (b.p. 35·7–36·7°), using 6 ml. for each extraction. The third extraction showed an optical density of 0, indicating that all of the prodigiosin had been removed from the culture. The light petroleum extracts were pooled and the optical density was recorded. Readings were taken at wavelength 570 mμ, the maximum absorption of prodigiosin in these tests. The results are recorded in Table 1. The optical density readings of the acidic acetone cultures (12 ml.) without prodigiosin are recorded in Table 2.

RESULTS

The effects of different concentrations of extracts of blue-green algae on the production of prodigiosin by *Serratia marcescens* during an incubation period of 72 hr. are shown in Table 1. The data represent the optical density of light petroleum extracts of prodigiosin from untreated cultures and from algal extract-treated cultures of *S. marcescens*. Prodigiosin extract from cultures of

S. marcescens treated with aqueous extract or aqueous filtrate from *Microcystis aeruginosa* or from *Nostoc rivulare* at concentration 0·2 ml./ml. medium had an optical density of 0, indicating the absence of pigment. Extract from untreated cultures of *S. marcescens* gave an optical density reading of 0·15. Both extracts from *Oscillatoria formosa* (0·2 ml./ml. medium) produced some inhibition of pigment formation, although not complete. Aqueous filtrates from *N. ellipsosporum*, *N. calcicola*, *N. punctiforme*, *Calothrix membranacea* and *Anabaena variabilis* produced partial inhibition. Aqueous extracts of *N. calcicola* and *N. punctiforme* also produced partial inhibition. Extracts from other species of blue-green algae tested produced no effect on cultures of *S. marcescens*.

In cultures of *Serratia marcescens*, in which complete or partial inhibition of pigment formation was produced APF remained effective for 96 hr. after which a gradual increase in pigmentation was observed. After incubation for 7 days there was little difference between the optical density of the experimental and control cultures. Tests on extracts from bacteria-contaminated cultures of algae gave the same results as extracts from bacteria-free cultures. Aqueous extracts or aqueous filtrate from bacteria isolated from the blue-green algae cultures exhibited no anti-pigmentation activities

Table 1. *Effect of blue-green algae extracts on the production of prodigiosin by* Serratia marcescens *during an incubation period of* 72 *hr.*

The data represent the optical density of light petroleum extracts of prodigiosin from algal extract-treated cultures and from untreated cultures of *S. marcescens.*

Source of extracts	Aqueous extract (ml./ml. medium)			Aqueous filtrate (ml./ml. medium)			Untreated cultures
	0·20	0·50	1·00	0·20	0·50	1·00	
Microcystis aeruginosa	0·00	0·03	0·04	0·00	0·03	0·05	0·15
Oscillatoria formosa	0·06	0·07	0·07	0·05	0·09	0·10	0·15
O. tenuis	0·15	0·16	0·16	0·15	0·15	0·16	0·16
Nostoc ellipsosporum	0·15	0·16	0·15	0·02	0·04	0·13	0·15
N. calcicola	0·14	0·09	0·15	0·03	0·08	0·14	0·16
N. rivulare	0·00	0·02	0·03	0·00	0·02	0·03	0·15
N. punctiforme	0·06	0·08	0·13	0·03	0·06	0·15	0·15
Calothrix membranacea	0·15	0·15	0·16	0·02	0·04	0·15	0·15
Anabaena variabilis	0·15	0·15	0·15	0·15	0·16	0·16	0·15
A. catenula	0·16	0·16	0·15	0·15	0·15	0·16	0·16
Tolypothrix distorta	0·15	0·15	0·16	0·15	0·16	0·15	0·15
Lyngbya sp.	0·16	0·16	0·15	0·15	0·15	0·15	0·16

It was important to determine whether the differences in the amount of pigment produced were caused by differences in the amount of growth of the bacteria. The data in Table 2 represent the optical density of algal extract-treated cultures and the untreated cultures of *Serratia marcescens* after the prodigiosin had been extracted. The optical density of these cultures was 0·31 (±0·02). It is evident, therefore, that the differences were not caused by differences in bacterial growth.

The optical density of sterile extract (0·02) and of sterile medium (0·20) was unchanged during the 72 hr. incubation period.

Table 2. *Effect of extracts of blue-green algae on the growth of* Serratia marcescens *during an incubation period of 72 hr.*

The data represent the optical density of algal extract-treated cultures and of untreated cultures of *S. marcescens* after the prodigiosin was extracted.

	Aqueous extract (ml./ml. medium)			Aqueous filtrate (ml./ml. medium)			
Source of extracts	0·20	0·50	1·00	0·20	0·50	1·00	Untreated cultures
Microcystis aeruginosa	0·31	0·31	0·30	0·28	0·28	0·31	0·31
Oscillatoria formosa	0·31	0·31	0·30	0·29	0·31	0·31	0·30
O. tenuis	0·33	0·32	0·32	0·31	0·31	0·31	0·33
Nostoc ellipsosporum	0·32	0·31	0·31	0·30	0·31	0·33	0·31
N. calcicola	0·33	0·32	0·31	0·32	0·31	0·31	0·32
N. rivulare	0·32	0·31	0·31	0·31	0·31	0·33	0·31
N. punctiforme	0·32	0·32	0·32	0·33	0·33	0·32	0·31
Calothrix membranacea	0·33	0·31	0·33	0·31	0·31	0·31	0·32
Anabaena variabilis	0·31	0·31	0·31	0·32	0·33	0·31	0·30
A. catenula	0·30	0·29	0·32	0·31	0·31	0·31	0·30
Tolypothrix distorta	0·31	0·31	0·31	0·32	0·30	0·32	0·31
Lyngbya sp.	0·31	0·31	0·33	0·30	0·32	0·31	0·31

DISCUSSION

The importance of using bacteria-free unialgal cultures in such an investigation is well known to the author. Although the Cyanophycean species used in this study were unialgal, only two, *Microcystis aeruginosa* Kütz. and *Oscillatoria formosa* Bory, were bacteria-free. Confirmation of results from impure algal cultures awaits the establishment of pure cultures, a project now being conducted in this laboratory. However, since the results obtained from pure and impure cultures of *M. aeruginosa* Kütz. or *O. formosa* Bory were the same, there is reason to believe that APF was produced only by the algae.

Various workers have shown that aqueous suspensions of *Microcystis aeruginosa* increase in toxicity as the cell walls rupture, either by natural decay or by quick-freezing in the laboratory. In the present series of tests, the toxic effects of aqueous suspensions of *M. aeruginosa* or *Nostoc rivulare* on mice and the anti-pigmentation activities of their extracts were increased when the organisms were disintegrated in a ball mill before their application. These results, and the fact that only those algae which were toxic to mice contained APF, indicate a close relationship between the poisonous material and the anti-pigmentation substance. These results suggest, therefore, the possibility of using the anti-pigmentation test as a rapid means of detecting minute quantities of toxic material in blue-green algae.

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REFERENCES

BUNTING, M. I. (1950). The effect of surface-active agents on color variation in aging populations of *Serratia marcescens*. *J. Bact.* **59**, 241.

BURKHOLDER, P. R. (1955). Culture collection of algae, Department of Bacteriology, University of Georgia, Athens, Georgia. (Mimeographed.)

CHU, S. P. (1942). The influence of mineral composition of the medium on the growth of plankton algae. I. Methods and culture media. *J. Ecol.* **30**, 284.

CRICHTON, P. V. & LAZARUS, A. S. (1948). The relationship between prodigiosin production and catalase activity. *J. Bact.* **56**, 375.

DE, P. K. (1939). The role of blue-green algae in nitrogen fixation in rice fields. *Proc. roy. Soc. Bot.* **127**, 121.

GERLOFF, F. C., FITZGERALD, G. P. & SKOOG, F. (1952). The mineral nutrition of *Microcystis aeruginosa*. *Amer. J. Bot.* **39**, 26.

GORYUNOVA, S. V. (1955). The occurrence of carnivorous blue-green algae. *Microbiology, Moscow*, **24**, 271.

HUBBARD, R. & RIMINGTON, C. (1950). The biosynthesis of prodigiosin, the tripyrrylmethene pigment from *Bacillus prodigiosus* (*Serratia marcescens*). *Biochem. J.* **46**, 220.

KREITLOW, K. W. (1942). Some effects of visible radiation on pigmentation of *Serratia marcescens*. *J. Bact.* **42**, 127.

LEFEVRE, M. H., JAKOB, J. & NESBET, M. (1952). Auto- et heteroantagonisme chez algues d'eau douce. *Ann. Sta. cent. Hydrobiol. appl.* **4**, 5.

NOVELLI, A. (1953). The influence of ultraviolet light and chlorophyll on the production of pigment by *Bacterium prodigiosum* (*Serratia marcescens*). *J. Bact.* **65**, 479.

PRINGSHEIM, E. G. (1949). *Pure Cultures of Algae*. Cambridge University Press.

STARR, R. C. (1956). Culture collection of algae at Indiana University. *Lloydia*, **19**, 129.

WEIL, A. J. (1952). Inhibition of pigment formation of *Serratia marcescens* by chloramphenicol, aureomycin and terramycin. *Proc. Soc. exp. Biol., N.Y.* **79**, 539.

WEINBERG, E. D. (1951). The influence of antibiotics and amino acids on the production of pigment by Serratia. *J. Bact.* **62**, 785.

WILLIAMS, R. P., GREEN, J. A. & RAPPOPORT, D. A. (1956). Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of prodigiosin. *J. Bact.* **71**, 115.

WURTZ, A. (1949). Propriétés particulières d'une fleur d'eau de Cyanophycée: *Microcystis aeruginosa* Kütz. *Bull. Soc. bot. Fr.* **96**, 50.

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The Infectivity of Purified and Partially Purified Preparations of Vaccinia and Cowpox Viruses

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SUMMARY: Twenty-three preparations of vaccinia virus and 5 of cowpox virus were examined by electron microscopy of spray droplets and by infectivity titrations at various stages of purification. Extraction of virus-containing tissues with a fluorocarbon compound was a useful early step in purification, but preparations of the highest purity were obtained by flocculation of the virus with M-NaCl. The average ratio of total particle count to infectivity for the vaccinia virus preparations was 12. The lowest observed ratio based on reliable particle counts was 3·4.

During inactivation of vaccinia virus, a very small, but constant, fraction of infectivity was observed to survive extremely high doses of gamma-radiation. In order to assess the likelihood of multiple reactivation being responsible for this persistence, it was necessary to know the ratio of infective units to total particle count in the type of virus preparation being irradiated. The two groups of published figures for vaccinia virus are not in close agreement, so several preparations of vaccinia virus, and a few of cowpox virus, were counted by electron microscopy and titrated for infectivity. The influence of various methods of purification on the preparations was also studied.

METHODS

Viruses. The vaccinia virus strains were all sublines of the Lister Institute vaccine strain. The *rabbit dermal virus* (RV) was a homogeneous line (Amies, 1938) in its 80th skin passage. *Chick embryo adapted virus* (LC) had been passed from 13 to 16 times in the chick embryo chorioallantois. *Tissue culture virus* (T) was taken from the first five batches of virus grown for smallpox vaccine preparation. This virus was a first passage of bacteriologically sterile RV in trypsinized chick embryo cells. The *cowpox virus* was a strain lyophilized in 1939 and recently cultivated. Its history is unknown, but it produced typical haemorrhagic lesions in the chick embryo chorioallantois after 3 days of incubation.

Diluent. All extractions and dilutions were made in McIlvaine's phosphate + citrate buffer (pH 7·2, 0·004M-phosphate). Treatments with trypsin were done with Armour's crystalline, salt-free product.

Extractions were made by mechanical disruption of the virus-infected tissues in a Servall 'Omnimix' homogenizer at 14,000 rev./min. for 5 min., with an ice-bath round the stainless steel vessel. Trifluorotrichloroethane (Gessler, Bender & Parkinson, 1956) was used in some of the extraction and purification procedures; it was obtained from Imperial Chemical Industries Ltd. as 'Arcton 63'.

Nitrogen was estimated by a micro-Kjeldahl method.

Methods of purification used are described under Results. The initial step in all the preparations was horizontal centrifugation of the extracts at 1500 *g* for 10 min., the virus-containing supernatant fluids being kept for further treatment.

Infectivity titrations were by pock count on the chick embryo chorio-allantois. Membranes were prepared and inoculated by the method of Westwood, Phipps & Boulter (1957). Infectivity titres (I) are expressed as infectious units/millilitre (i.u./ml.).

Total particle counts (*T*) were made with the electron microscope by the spray drop method of Williams & Backus (1949), which was fully described by Luria, Williams & Backus (1951). The polystyrene latex spheres used as indicator particles were generously given by The Dow Chemical Corporation, Midland, Michigan, U.S.A., from their batch no. LS-055-A. The absolute count of these particles/ml. suspension was determined from its dry weight, the density of the latex (1·05 g./ml.) and their diameter. This latter measurement was made in terms of an electron transparent aluminium replica of a standard grating on which the particles were mounted. An independent determination was also made in terms of the diameter of the particles in the original batch of latex (580-G) which was well established as 0·26 μ, the mean of many different independent determinations made in various laboratories reported by Gerould (1950). Both methods gave the diameter of 0·16 μ for the latex used. This differs considerably from the figure supplied by the manufacturers (0·19 μ) which was measured on a nitrocellulose replica (Bradford & Vanderhoff, 1955). As nitrocellulose can contract considerably when the replica is mounted on its support we have preferred our figure of 0·16 μ. The use of the larger figure would decrease the particle counts cited below by a factor of 0·6.

A sufficient number of the latex and virus particles was counted in each preparation to give a standard error of *c*. 10% in their ratio (this requires a count of 200 particles of each kind when the ratio is one).

RESULTS

Influence of 'Arcton 63' on virus yield and purity

The use of 'Arcton 63' in the preliminary extraction of the virus was suggested by Gessler *et al.* (1956). The efficiency of repeated extraction in Arcton 63 was tested by extracting several rabbit dermal pulps in 20 ml. buffer with 1 ml. Arcton/g. of pulp. The supernatant of the first extraction was kept and the Arcton-containing deposit was re-extracted after the addition of 20 ml. of fresh buffer. The second extraction may set free as much as 15% of the first yield (Table 1). The average ratio of particles to infective units in the first extractions was 15.

The efficiency of Arcton 63 as a first step in purification was tested by extracting equal weights of dermal pulp in buffer and in buffer+Arcton 63 (1 ml./g. pulp). Homogenizing with Arcton 63 reduced the total nitrogen content of the centrifuged extract by about 30% (Table 2). Compared with the opaque, milky supernatants of the buffer extracts, the Arcton extracts were translucent. The titres of the extracts were the same whether Arcton 63 was used or not. However, the removal of as much as 30% of impurity during the extraction, without measurable loss of virus, is a very useful start in a purification process.

Table 1. *Efficiency of repeated extraction in Arcton 63*

Prep. no.	I_1 1st extraction	I_2 2nd extraction	I_2/I_1	T 1st extraction	T/I_1
1	$8{\cdot}5 \times 10^9$	$1{\cdot}3 \times 10^9$	0·15	$8{\cdot}9 \times 10^{10}$	10·5
2	$3{\cdot}3 \times 10^9$	$3{\cdot}6 \times 10^8$	0·11	$6{\cdot}2 \times 10^{10}$	18·8
3	$4{\cdot}36 \times 10^9$	$2{\cdot}75 \times 10^8$	0·06	$7{\cdot}2 \times 10^{10}$	16·5
4	$2{\cdot}96 \times 10^9$	$4{\cdot}75 \times 10^8$	0·16	$5{\cdot}0 \times 10^{10}$	16·9

I = titre: infectious units/ml.; T = total particle count.

Table 2. *Influence of Arcton 63 on virus yield and purity*

Pulp no.	Extracted in	I	N_2 (mg./ml.)
70	Buffer	$2{\cdot}5 \times 10^9$	0·75
	Buffer+Arcton	$2{\cdot}5 \times 10^9$	0·52
71	Buffer	$3{\cdot}6 \times 10^9$	0·66
	Buffer+Arcton	$3{\cdot}2 \times 10^9$	0·43
73	Buffer	$7{\cdot}0 \times 10^9$	1·08
	Buffer+Arcton	$6{\cdot}0 \times 10^9$	0·71

Purification of vaccinia and cowpox viruses by various methods

Dermal virus. The virus was extracted from dermal pulp in buffer, and after initial centrifugation, purification was undertaken by several methods (Table 3). RV80/a, b and c were dealt with by one, two and three cycles, respectively, of differential centrifugation. A cycle consisted of sedimentation of the virus in an angle rotor (28° from vertical) at 2600 *g* for 60 min., and, after resuspension in fresh buffer by stirring and gentle pipetting with a wide bore (1·5–2·0 mm.) Pasteur pipette, deposition of debris by horizontal centrifugation at *c.* 1500 *g* for 5 min. At the final resuspension, the preparation was concentrated tenfold or more. Table 3 records the particle counts and infectivities of the various preparations. The vaccinated area of skin of RV80/a was inadvertently swabbed with 20% (v/v) ethanol in water immediately before harvesting, instead of with 1% 'Hibitane'. McClean (1945) showed that ethanol destroyed the infectivity of vaccinia virus, and the high ratio of particles to infectivity found in this preparation may well be a reflexion of this. In the other two preparations the ratio was less than 10.

Plate 1, fig. 1, shows the fuzzy appearance of the virus which indicates the presence of soluble impurities in the preparation. Dawson & McFarlane (1948) produced very pure virus by flocculating their preparations with M-NaCl, washing, and then resuspending the virus by ultrasonic vibration. Section *b* of Table 3 displays the results obtained when the clarified supernatant fluid of a dermal pulp extract was divided into four portions of equal volume, two of which were then salt flocculated. After this treatment the preparations contained far less extraneous material as judged from electron micrographs and the particles were more sharply defined (Pl. 1, fig. 2). Although (according to Dawson & McFarlane, 1948) about 40% of the virus is lost in the supernatant after salt flocculation, not only is the infectivity of the remaining virus undiminished, but there is a suggestion that it is increased.

Table 3. *Influence of purification procedures on infectivity and particle count of rabbit dermal vaccinia virus*

	Prep. no.	Treatment	*I*	*T*	*T/I*
a	RV80/a	(Eth.) d.c.	$4{\cdot}5 \times 10^{8}$ (±80%)	$5{\cdot}8 \times 10^{10}$	129 (±81%)
	RV80/b	d.c. ×2	$1{\cdot}4 \times 10^{10}$ (±31%)	$1{\cdot}1 \times 10^{11}$	7·8 (±33%)
	RV80/c	d.c. ×3	$2{\cdot}7 \times 10^{10}$ (±19%)	$1{\cdot}9 \times 10^{11}$	7·0 (±22%)
b	A1	A; d.c.; NaCl; u.s.	$2{\cdot}4 \times 10^{9}$ (±28%)	*c.* 3×10^{9}	*c.* 1·2
	A2	A; d.c.; u.s.	$2{\cdot}8 \times 10^{9}$ (±37%)	$2{\cdot}6 \times 10^{10}$	9·3 (±39%)
	B1	B; d.c.; NaCl; u.s.	$9{\cdot}5 \times 10^{9}$ (±26%)	$4{\cdot}2 \times 10^{10}$	4·4 (±28%)
	B2	B; d.c.; u.s.	$4{\cdot}6 \times 10^{9}$ (±23%)	$4{\cdot}9 \times 10^{10}$	10·7 (±25%)

Eth. = swabbed with 20% (v/v) ethanol in water; d.c. = differential centrifugation; A = extracted with Arcton 63; B = extracted with buffer; NaCl = flocculated with M-NaCl; u.s. = resuspended by ultrasonic vibration.

Table 4. *Influence of trypsin on the infectivity of chick embryo vaccinia virus*

Prep. no.	Treatment	*I*	*T*	*T/I*
L13	d.c.	$2{\cdot}4 \times 10^{9}$ (±29%)	$4{\cdot}4 \times 10^{10}$	18·4 (±31%)
L14 i	d.c.	$7{\cdot}4 \times 10^{9}$ (±58%)	$3{\cdot}5 \times 10^{10}$	4·7 (±59%)
L14 ii	d.c.	$7{\cdot}3 \times 10^{9}$ (±14%)	$2{\cdot}4 \times 10^{10}$	3·4 (±17%)
L15B	d.c.; B	$1{\cdot}1 \times 10^{10}$ (±35%)	$1{\cdot}6 \times 10^{11}$	14·2 (±36%)
L15E	d.c.; E	$8{\cdot}0 \times 10^{9}$ (±6%)	$1{\cdot}8 \times 10^{11}$	22·5 (±12%)
L16B	d.c.; B	$1{\cdot}4 \times 10^{10}$ (±43%)	$1{\cdot}4 \times 10^{11}$	10·0 (±45%)
L16E	d.c.; E	$2{\cdot}1 \times 10^{10}$ (±31%)	$2{\cdot}6 \times 10^{11}$	12·4 (±33%)

d.c. = differential centrifugation; B = incubation with buffer for 30 min. at 37°; E = incubation with 0·025% trypsin for 30 min. at 37°.

Chick embryo adapted virus. Table 4 shows the results obtained by purification of chick embryo virus by centrifugation and a combination of centrifugation and enzymic methods. Chick embryo adapted virus cannot be flocculated by M-NaCl. The reason for this is unknown. Protein impurities may, however, be sensibly diminished by treatment with trypsin (Smadel & Wall, 1937). The results of these experiments suggest that trypsin is not harmful to the virus.

Tissue culture virus. The harvested cultures were disrupted in the Servall 'Omnimix'. Cell debris and virus were then sedimented in an angle centrifuge at 2600 *g* for 60 min. and the deposit resuspended in fresh buffer to give a volume concentration of about 20-fold. To overcome clumping of virus in the cell debris the suspension was homogenized for 30 sec. in the 'Omnimix' before titration. No other procedure was used to purify the virus. Pl. 1, fig. 3, shows the degree of contamination with cellular components compared with that of a rabbit virus preparation given one cycle of differential centrifugation (Pl. 1, fig. 4). The ratio of particles to infectivity (Table 5) does not differ greatly from that of partially purified rabbit dermal virus (Table 1).

Table 5. *Particle count/infectivity ratio of tissue culture vaccinia virus*

Prep. no.	*I*	*T*	*T/I*
T1	$1{\cdot}0\times10^{10}$ (±25%)	$1{\cdot}25\times10^{11}$	12·5 (±27%)
T2	$8{\cdot}9\times10^{9}$ (±16%)	$1{\cdot}3\times10^{11}$	14·6 (±19%)
T3	$7{\cdot}1\times10^{9}$ (±59%)	$8{\cdot}1\times10^{10}$	11·4 (±60%)
T4	$8{\cdot}5\times10^{9}$ (±16%)	$9{\cdot}3\times10^{10}$	10·9 (±19%)
T5	$7{\cdot}0\times10^{9}$ (±9%)	$1{\cdot}3\times10^{11}$	18·6 (±13%)

Cowpox virus. A few preparations only of this virus were examined. The results (Table 6) are in general agreement with those of Dumbell, Downie & Valentine (1957) as far as the high ratio of particles to pock-producing infective units is concerned, but the number of preparations studied was too small to allow further comment.

Table 6. *Influence of trypsin on cowpox virus yield and infectivity*

Prep. no.	Treatment	*I*	*T*	*T/I*
C3	d.c.	$3{\cdot}1\times10^{9}$ (±24%)	$4{\cdot}7\times10^{11}$	150 (±26%)
C4B	d.c.; B	$3{\cdot}5\times10^{9}$ (±36%)	$1{\cdot}9\times10^{11}$	54·5 (±38%)
C4E	d.c.; E	$2{\cdot}4\times10^{9}$ (±17%)	$1{\cdot}9\times10^{11}$	78 (±20%)
C5B	d.c.; B	$2{\cdot}6\times10^{9}$ (±17%)	$1{\cdot}9\times10^{11}$	72 (±20%)
C5E	d.c.; E	$1{\cdot}3\times10^{9}$ (±38%)	$1{\cdot}6\times10^{11}$	127 (±39%)

d.c. = differential centrifugation; B = incubation with buffer for 30 min. at 37°; E = incubation with 0·025% trypsin for 30 min. at 37°.

DISCUSSION

Judged by their electron microscope appearances, preparations purified by salt flocculation contained less extraneous material than those in which salt flocculation was not used. Arcton 63 extraction (Gessler *et al.* 1956) is a useful addition to the methods available for purification of vaccinia virus, but as Epstein (1958) showed, even five successive extractions with Arcton 63 may not entirely remove non-virus material. He found free deoxyribonucleic acid in ultracentrifugates of vaccinia virus extracted from infected chorioallantoic membranes. The appearance of the virus in our electron micrographs (Pl. 1, figs. 1, 2) suggests that flocculation with M-NaCl is always a worthwhile final step in the procedure when highly purified vaccinia virus is wanted. The virus yields shown in Table 1 indicate that more than two extractions of virus-containing material with Arcton 63 are not worthwhile.

The ratios of almost unity of total to infective vaccinia virus particles

(T/I) found by Overman & Tamm (1956) are considerably lower than those reported here. However, we suspect that their method of estimating the total count may well have given too low a figure because some virus was not picked up on the supports and some was obscured by debris. In view of such criticisms, an attempt was recently made to demonstrate the accuracy of the sedimentation technique (Sharp & Overman, 1958). It appears that the method does give a reliable estimate of the number of vaccinia particles in a sample provided this has been adequately diluted. However, the dilutions used by Overman & Tamm (1956) when making their counts were lower than those now found to be necessary. This subsequent work confirms the opinion that the counts recorded by Overman & Tamm were in error. The figures of Dumbell *et al.* (1957) are more in accord with ours. However, despite a close correspondence between their T/I ratios and ours for cowpox virus, based on the pock count titre, their vaccinia virus T/I ratios are somewhat higher than ours; excluding their trypsin-treated preparations, their ratio was 18. The average for all our preparations was 12 and in several of these the pock count, for reasons connected with the preparation of the membranes, was certainly too low. This suggests that the technique of chorioallantoic membrane inoculation of Dumbell *et al.* is probably less sensitive than that of Westwood *et al.* (1957) which we used.

Unlike Dumbell *et al.* (1957), we failed to find any substantial deleterious effect of trypsin on the infectivity of vaccinia virus. However, the results obtained with cowpox virus do not exclude an inactivating effect of trypsin on the virus. The inactivation Dumbell *et al.* observed could have been due to their use of 'Difco' trypsin 1/250. Pirie (1935) showed that dried pancreas contains substances soluble in light petroleum which can destroy the infectivity of vaccinia and some other viruses. One of us (C. K.) found that 'Difco' trypsin (a preparation of dried pig pancreas) behaved like Pirie's preparations, and that thorough extraction of this material with petroleum ether removed the virucidal substances without significantly altering its tryptic activity.

There was little, if any, inactivation of vaccinia virus by the methods of purification used in this work. On several occasions when the clarified extracts were titrated as well as the final virus suspensions, recovery was in the region of 90%. Nevertheless, the fact that ratios of total particle count to infectivity reliably close to unity were not obtained might be due to either the presence in all fresh preparations of 'incomplete' virus, or to the use of titration methods that are less than 100% efficient. Unlike the influenza viruses, vaccinia virus has no known attribute other than infectivity by which it may be assayed biologically. In the absence of any evidence in its favour, the 'incomplete' virus hypothesis will not be discussed. It remains, therefore, to consider possible causes of inefficiency in the available titration methods.

The pock-counting method of titrating vaccinia virus is probably the most sensitive one in general use. It has, however, the inherent limitation imposed by the slow rate of adsorption of virus to sensitive cells. This is due directly to the size of the virus: being relatively large it diffuses very slowly and therefore it is improbable that all the infectious particles in a given dose of virus will meet susceptible cells before appreciable loss of infectivity has occurred at 37°. Boulter (1957) showed that in dilute McIlvaine buffer (the diluent used in these experiments) approximately a quarter of the virus was inactivated after 2 hr. incubation at 37°. Because of its size, maximum adsorption of the virus may be expected to take longer than 2 hr., with a corresponding increase in the proportion inactivated. Investigation of a medium which protects vaccinia virus from heat inactivation might thus give a lower ratio of total to infectious particles than that obtained with suspensions in dilute buffer.

Even when the hazard of heat-inactivation is removed, the chorionic cells of the chorioallantois may not, however, be equally susceptible to the virus either because of natural factors or because of varying degrees of invisible trauma suffered by the membrane during the dropping procedure. T/I ratios close to but significantly greater than unity are therefore to be expected even if virus preparations contain no inactive particles.

REFERENCES

Amies, C. R. (1938). The production of homogeneous suspensions of vaccinia elementary bodies and the histology of the associated skin lesions. *J. Path. Bact.* **47**, 205.

Boulter, E. A. (1957). The titration of vaccinial neutralizing antibody on chorioallantoic membranes. *J. Hyg., Camb.* **55**, 502.

Bradford, E. B. & Vanderhoff, J. W. (1955). Electron microscopy of monodisperse latexes. *J. appl. Phys.* **26**, 864.

Dawson, I. M. & McFarlane, A. S. (1948). Structure of an animal virus. *Nature, Lond.* **161**, 464.

Dumbell, K. R., Downie, A. W. & Valentine, R. C. (1957). The ratio of the number of virus particles to infective titer of cowpox and vaccinia virus suspensions. *Virology*, **4**, 467.

Epstein, M. A. (1958). An investigation into the purifying effect of a fluorocarbon on vaccinia virus. *Brit. J. exp. Path.* **39**, 436.

Gerould, C. H. (1950). Comments on the use of latex spheres as size standards in electron microscopy. *J. appl. Phys.* **21**, 183.

Gessler, A. E., Bender, C. E. & Parkinson, M. C. (1956). A new and rapid method for isolating viruses by selective fluorocarbon deproteinization. *Trans. N.Y. Acad. Sci.* **18**, 701.

Luria, S. E., Williams, R. C. & Backus, R. C. (1951). Electron micrographic counts of bacteriophage particles. *J. Bact.* **61**, 179.

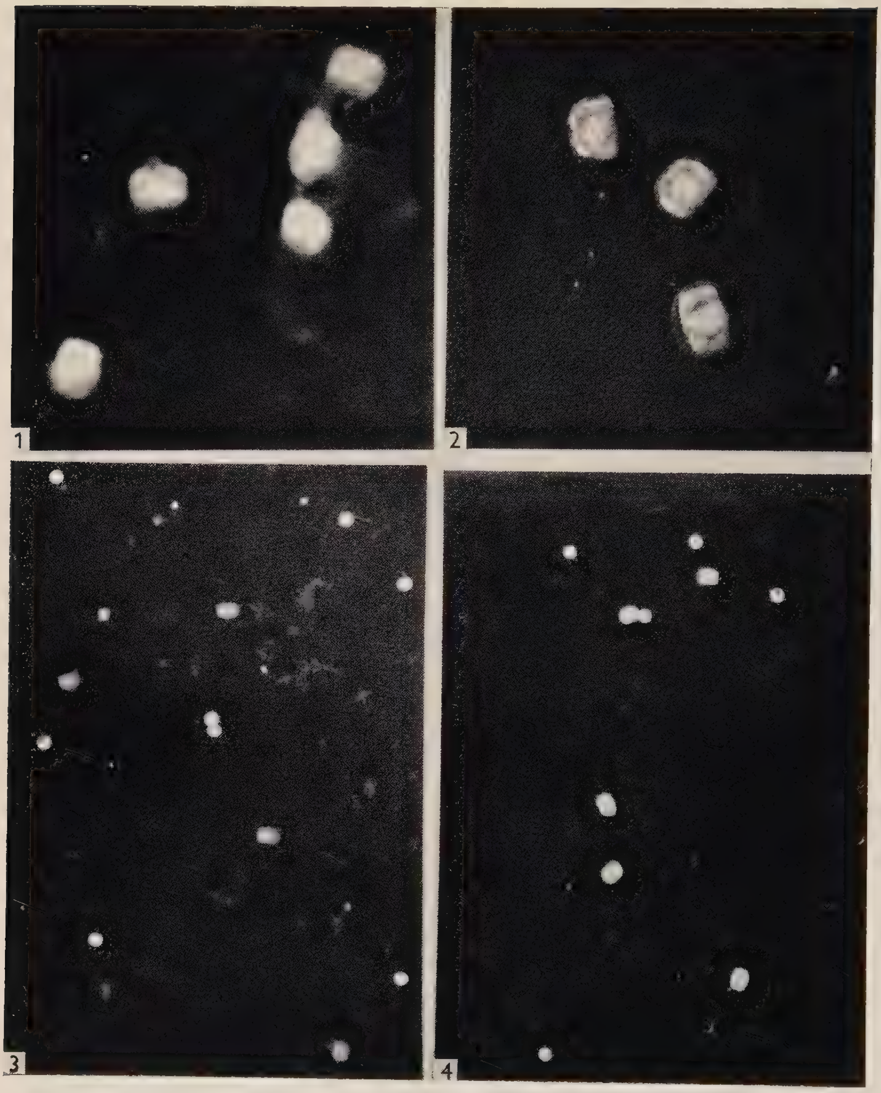
McClean, D. (1945). The antigenicity of vaccinia virus inactivated with alcohol. *J. Path. Bact.* **57**, 261.

Overman, J. R. & Tamm, I. (1956). Equivalence between vaccinia particles counted by electron microscopy and infectious units of the virus. *Proc. Soc. exp. Biol., N.Y.* **92**, 806.

Pirie, A. (1935). The effect of extracts of pancreas on different viruses. *Brit. J. exp. Path.* **16**, 497.

Sharp, D. G. & Overman, J. R. (1958). Enumeration of vaccinia virus particles in crude extracts of infected tissues by electron microscopy. *Proc. Soc. exp. Biol., N.Y.* **99**, 409.

Smadel, J. E. & Wall, M. J. (1937). Elementary bodies of vaccinia from infected chorio-allantoic membranes of developing chick embryos. *J. exp. Med.* **66**, 325.



C. Kaplan & R. C. Valentine—Purified pox viruses. Plate 1

(*Facing p.* 619)

Westwood, J. C. N., Phipps, P. H. & Boulter, E. A. (1957). The titration of vaccinia virus on the chorio-allantoic membrane of the developing chick embryo. *J. Hyg., Camb.* **55**, 123.

Williams, R. C. & Backus, R. C. (1949). Macromolecular weights determined by direct particle counting. I. The weight of the bushy stunt virus particle. *J. Amer. chem. Soc.* **71**, 4052.

EXPLANATION OF PLATE

The virus preparations were fixed in 2 % formaldehyde. Electron micrographs were taken after lightly shadowing the specimens with platinum.

Fig. 1. Partially purified vaccinia virus showing the fuzzy appearance caused by soluble impurities. × 50,000.

Fig. 2. Virus purified further by salt flocculation. There is less extraneous material and the particles are more sharply defined. × 50,000.

Fig. 3. Virus from tissue culture. Part of a spray droplet used in making the virus particle count. Despite some contamination with cell debris the count of this area is clearly 4 virus and 8 latex particles. × 19,000.

Fig. 4. Virus from rabbit dermis. Part of a spray droplet showing 5 virus and 5 latex particles. There is little debris. × 19,000.

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ROUNTREE, P. M. (1959). *J. gen. Microbiol.* **20**, 620–633

Changes in the Phage-Typing Patterns of Staphylococci Following Lysogenization

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SUMMARY: Staphylococcal strains which showed a range of reactions with the phages 47C, 52, 52A, 80 and 81 were examined for changes in phage sensitivity following artificial lysogenization. The lysogenic phages used were isolated from strains of three different phage-typing patterns and differed in serology. The changes in phage reactions thus produced included: (1) immunity to the B phages, 52, 52A and 80 produced by a B phage; (2) immunity to the A phage 81 produced by a serologically unrelated F phage; (3) immunity to phage 47C and sensitivity to phages 52 and 52A produced by a number of A phages. The mechanisms whereby a variety of typing patterns may be obtained within a related group of staphylococci are discussed.

In their study of the stability of phage-typing patterns in staphylococci, Williams & Rippon (1952) used, among various sets of cultures, sequential ones from single sources such as nasal carriers, cultures from different sites on a single subject and cultures from different colonies from one primary culture plate. They found differences between members of these sets of cultures which indicated that some change in typing pattern occurred from day to day, both *in vivo* and *in vitro*. How much of this change was due to differences in the composition of the media used for typing and how much to changes in phage sensitivity of the staphylococci used was difficult to assess. However, their proposed rules for regarding strains of staphylococci as identical with or different from one another indicate that loss or gain of one 'strong reaction' may not be sufficient for regarding strains as different.

Smith (1948), Lowbury & Hood (1953) and Rountree (1956) showed that changes in phage-typing pattern followed artificial lysogenization; these changes comprised losses in phage sensitivity and therefore indicated immunity in Lwoff's sense (1953). How frequent such changes may be *in vivo* is not known, but obviously they may have some importance in the interpretation of phage-typing results.

Following the identification in Australia of a new phage type of staphylococcus lysed only by a new phage 80 (Rountree & Freeman, 1955) the origin of this new strain became of interest. Phage 80, a B phage serologically, was 'adapted' from phage 52A, of which it appears to be a virulent mutant since attempts to set up lysogenic systems with it have been unsuccessful. Strains of staphylococci lysed by both the B phages, 52 and 52A are also lysed by phage 80. Bynoe, Elder & Comtois (1956) encountered in Canada a similar strain and identified it with a new phage 81, which is serologically A and also appears to be a virulent phage. The phage-typing pattern of the propagating strains of phages 80 and 81 is 80/81.

In recent years, strains have been isolated in Australia which type as 80/81, as 80 alone or as 81 alone. Strains typing as 52/52A/80/81 are also found and may be fully or partially sensitive or resistant to phage 47C. The staphylococcus responsible for the Bundaberg disaster (Kellaway, McCallum & Tebbutt, 1928) has the phage pattern 52/52A/80/81.

Since it was known that strains typing as 52/52A (and also presumably sensitive to phages 80 and 81) had been isolated before any known occurrence of 80/81 strains, the hypothesis that the 80/81 pattern might have been derived from 52/52A/80/81 by lysogenization with a phage giving immunity to phages 52 and 52A was attractive. Preliminary investigations produced no evidence to support this hypothesis since no lysogenic B phages were found in 80/81 strains. It was then decided to investigate the lysogenicity of a series of strains showing various combinations of reactions with the phages 47C, 52, 52A, 80 and 81 and to transfer their lysogenic phages to other strains. If the phage-typing patterns within this series of strains depended on prophage immunity, then artificially lysogenized strains should resemble the strains used as sources of the prophages.

The strains selected for investigation on the basis of their typing patterns with the phages at routine test dilution were next examined with the undiluted phages. It then became apparent that they possessed characteristic patterns of 'inhibition' by high-titre phage filtrate. Williams & Rippon (1952) drew attention to this 'inhibitory' effect of high titre phage filtrates and showed that the 'inhibitory' agent was no longer detectable in filtrates from which the phage had been removed by adsorption, by filtration or by neutralization with antisera. Unpublished observations in this laboratory indicate that the 'inhibition' is probably due to phage growth in populations of partly resistant staphylococci; it does not appear to be correlated with the prophage state of the cocci.

METHODS

Staphylococci. The strains of staphylococci used came from a variety of sources. The propagating strains of phages 80 and 81 are referred to as PS80 and PS81 respectively. PS81 and phage 81 were obtained from the Staphylococcus Reference Laboratory, Colindale, London, England. Strain Bundaberg, referred to as B′b, was obtained from Dr J. Graydon of the Commonwealth Serum Laboratories, Victoria, Australia; in colonial appearance it corresponds to its original description, save for the loss of haemolysin. Other strains which will be referred to by number were selected from cultures sent from a number of places for routine phage typing. The phage-typing patterns and 'inhibition' patterns of representative strains are shown in Table 1. In addition to these strains, a number of others of similar patterns were used and will be mentioned in the text.

Preparation of phage stocks. The methods of preparing phage stocks on agar and of their titration have been described (Rountree, 1949*a*, 1952). Some phage stocks were also prepared by induction of lysogenic strains by ultraviolet irradiation (Gorrill & Gray, 1956).

Lysogenicity. Strains of staphylococci were tested for lysogenicity by growing them in nutrient broth at 37° for 2–3 hr., centrifuging and plating the supernatant fluids on appropriate indicator strains. PS 80 was the indicator strain of choice for many of the lysogenic phages.

Table 1. *Range of phage reactions in a series of staphylococci lysed by one or more of the phages* 47 C, 52, 52 A, 80 *and* 81

Strain no.	Place of isolation	Phage pattern at RTD*	'Inhibition' pattern with strong phage
8680	Sydney	47 C/52/52 A/80/81	—
6338	Coff's Harbour, N.S.W.	47 C†/52/52 A/80/81	—
684	Bairnsdale, Victoria	52/52 A/80/81	—
Bundaberg	Bundaberg, Queensland	52/52 A/80/81	—
PS 80	Sydney	80/81	47 C/52/52 A
PS 81	Canada	80/81	47 C/52/52 A
863	Sydney	80	47 C/52/52 A
864	Sydney	81	47 C/52/52 A

* RTD = routine test dilution. † Weak reaction only.

The antigenic character and host ranges of these phages after propagation on their indicator strains were determined by methods previously described (Rountree, 1949*b*, 1956). Determinations of host range were carried with the propagating strains of Wilson & Atkinson (1945) and are, in general, expressed as ratios of number of plaques/ml. on the propagating strain of the particular phage stock to plaques/ml. on the indicator strains. Phages obtained from lysogenic strains are designated by the number or letter of their strain of origin followed by the number of the strain used for their propagation; e.g. phage B′b/80 is a lysogenic phage from strain Bundaberg grown in PS 80.

Lysogenization. All phages used for lysogenization were filtered through Gradocol membranes of 0·8 μ APD (average pore diameter) and tested for sterility before use. Strains to be lysogenized were grown in broth and then flooded on the surface of agar plates, dried and the undiluted phage spread on the surface of the plates. After incubation overnight at 37°, a small quantity of the secondary bacterial growth in the area of confluent phage lysis was streaked on to agar plates in such a way as to obtain separate colonies. Single non-eroded colonies from the edge of the inoculum were transferred to broth and incubated. No estimate has been made of the proportion of lysogenic responses obtained, but all the phages readily established lysogenic systems.

The lysogenized cultures were tested for the presence of lysogenic phage by plating their supernatant fluids on the homologous phage-sensitive strain; for contaminating lytic phage by flooding on agar and after incubation inspecting the resultant lawn for plaques; for resistance to the lysogenizing phage and for alterations in phage-typing pattern.

Lysogenized strains have their phage indicated in parentheses after the strain letter or number, e.g. PS 80 (B′b) is lysogenized with a phage derived from strain Bundaberg.

RESULTS

Alterations in phage sensitivity by lysogenization with phage 864

Staphylococcus 864, lysed only by phage 81, was 'inhibited' by phages 47C, 52 and 52A and gave no reaction with undiluted phage 80; that is, it was immune to phage 80. It was lysogenic and its phage, 864, serologically B, could be propagated on strains sensitive to phage 80. A preparation of this phage made in strain 863 (phage 864/863) was used to lysogenize seven strains of various phage patterns. Table 2 records the changes in phage sensitivity that occurred. Six strains originally fully sensitive to phage 80 became immune. In the case of strain Bundaberg, the phage sensitivity was changed to 'inhibition'. Reactions with the related B phages, 52 and 52A, were either abolished or changed to 'inhibition'. Whether immunity or 'inhibition' followed lysogenization was not correlated with the original typing pattern

Table 2. *Effect of lysogenization with phages* 863 *and* 864 *on phage-sensitivity patterns of staphylococci*

	Phages				
Strain no.	47C	52	52A	80	81
	Reactions before lysogenization				
863	( )	( )	( )	++++	–
864	( )	( )	( )	–	++++
PS80	( )	( )	( )	++++	++++
PS81	( )	( )	( )	++++	++++
6351	( )	( )	( )	++++	++++
B'b	–	++++	++++	++++	++++
6338	+	++++	++++	++++	++++
6922	+	++++	++++	++++	++++
8680	++++	++++	++++	++++	++++
	Reactions after lysogenization with phage 864				
PS80	( )	–	–	–	++
PS81	( )	–	–	–	++++
6351	( )	–	–	–	++++
B'b	–	( )	( )	( )	( )
6338	( )	–	–	–	++++
6922	++	( )	( )	–	++++
8680	( )	–	–	–	++++
	Reactions after lysogenization with phage 863				
PS80	( )	( )	( )	++++	–
PS81	( )	++++	++++	++++	–
or	( )	( )	( )	++++	–
6351	( )	++++	++++	++++	–
or	( )	( )	( )	++++	–
B'b	–	++++	++++	++++	–
6338	( )	++++	++++	++++	–
6922	++	++++	++++	++++	–
8680	( )	++++	++++	++++	–

( ) = 'inhibition' with undiluted phage; ++++ = confluent lysis with phage at routine test dilution (RTD); ++ = 100–200 plaques with phage at RTD; + = < 100 plaques with phage at RTD; – = no reaction with undiluted phage.

of the strain. After lysogenization there was no alteration in sensitivity to the A phage 81, with the exception of strain Bundaberg which showed 'inhibition'. Similarly, there was no immunity to the A phage 47C, although reactions might be decreased in sensitivity.

The results showed that sensitivities to the B phages 52, 52A and 80 were closely linked and that the introduction of a B prophage resulted in partial or complete resistance to all three phages. When the typing patterns at the routine test dilutions only of phages 52, 52A and 80 are taken into account, all the lysogenized strains and strain 864 are identical. Phage 864/863 therefore in its prophage state blocked the multiplication of phage 80 in previously sensitive cocci; it is not, however, identical with phage 80. It behaved as a temperate and not as a virulent phage and its host range (Table 3) differed in several respects from that of our stock of phage 80. In particular, it did not lyse propagating strains 31/44, 42C or 52, and it produced 100 times fewer plaques on propagating strain 52A. It was concluded that the presence of this prophage was responsible for immunity to phage 80 in strain 864.

Table 3. *Host ranges of phage* 80 *and phage* 864/863

	Phage 80	Phage 864/863
Phage titre/ml.:	9×10^8	$2{\cdot}5 \times 10^8$
Indicator strain	Ratio: titre on propagating strain to titre on indicator strain	
6	—	1,000
31/44	1,000	—
42C	10,000	—
44A	1,000	1,000
47	500	200
47C	1,000	200
52	100	—
52A	150	10,000
80	1	1

Alterations in phage sensitivity by lysogenization with phage 863

Staphylococcus 863 was lysed only by phage 80, it was 'inhibited' by undiluted phages 47C, 52 and 52A and was immune to phage 81. It was lysogenic, and its phage 863 could be propagated in strains lysed by phage 81. When phage 863/864 was prepared it proved to be serologically F. The same strains used for lysogenization with phage 864 were lysogenized with phage 863/864 and the resulting changes in phage sensitivity are shown in Table 2. Lysogenization with this F phage resulted in immunity to phage 81 in all seven strains. There was little or no effect on reactions with phage 47C. In general, there was little effect on reactions with the B phages, but two anomalous cultures PS81 (863) and 6351 (863) were isolated which will be discussed in a later section of this paper.

It was concluded that the presence of the F phage in the prophage state in cocci of strain 863 was responsible for the immunity to the A phage 81. Since this was a surprising result, the stocks of phage 81 were confirmed as being serologically A. There was no crossing in neutralization tests with anti-A and anti-F sera (Rountree, 1949*b*). Strain 863 therefore provides an exception to previous findings that there is no cross-immunity between phages of unrelated antigenic structure. It should be noted, however, that the host ranges of phages 81 and 863/864 are very similar, the main differences being lack of minor reactions by phage 863 on the propagating strains 7 and 42C.

The effect of lysogenization with A phages on 80/81 *strains*

In two cultures, PS81 (863) and 6351 (863) shown in Table 2, lysogenization with the F phage 863 had apparently resulted in a change of sensitivity to phages 52 and 52A. Instead of being 'inhibited' by high-titre phages, the lysogenized cocci were as sensitive as the propagating strains of phages 52 and 52A. This was not however a constant feature of the lysogenized clones of these two strains since others still showed 52/52A 'inhibition'.

Table 4. *Effect of lysogenization with* A *phages*, B′b *and* 684, *on the phage typing patterns of* 80/81 *strains*

	Phages				
Strain no.	47C	52	52A	80	81
	Reaction				
PS80, PS81, 6351, 6352, 6402, 9183	( )	( )	( )	++++	++++
PS80 (B′b)	–	++++	++++	++++	++++
PS80 (684)	–	++++	++++	++++	++++
PS81 (B′b)	–	++++	++++	++++	++++
PS81 (684)	–	++++	++++	++++	++++
6351 (B′b)	–	++++	++++	++++	++++
6351 (684)	–	++++	++++	++++	++++
6352 (B′b)	–	++	++	++	++
6352 (684)	–	++	++	++	++
6402 (B′b)	–	++++	++++	++++	++
6402 (684)	–	++++	++++	++++	++++
9183 (B′b)	–	++++	++++	++++	++++
9183 (684)	–	++++	++++	++++	++++

Dr Joan Rippon, in a personal communication, stated that the transfer of certain A prophages to strains typing as 80/81 resulted in strains which typed as 52/52A/80/81. A search for similar lysogenic phages was made among our strains and Dr Rippon's observations confirmed. Two strains, Bundaberg and 684, which type as 52/52A/80/81 were found to be lysogenic for A phages to which strains typing as 80/81 are fully sensitive. These two phages, B′b and 684, were propagated in PS80 and used to lysogenize six strains typing as 80/81. All the lysogenized clones typed as 52/52A/80/81; in addition, their 'inhibition' with phage 47C disappeared and they became immune to this phage (Table 4). The presence or absence of reactions with phage 47C was therefore used as an index of the presence or absence in strains typing as 52/52A/80/81 of an A prophage capable of 'converting' 80/81 strains to 52/52A/80/81. The phages which produce this change in phage sensitivity will be referred to as ‘converting’ phages and the change termed ‘conversion’ without implying the exact mechanism involved. Five further strains typing as 52/52A/80/81 were found to be lysogenic for these ‘converting’ phages while eight strains typing as 52/52A/80/81 but showing ‘inhibition’ with undiluted phage 47C were devoid of lysogenic activity when they were plated on PS80.

Characters of the ‘converting’ phages

Five of the ‘converting’ phages, namely, B′b, 684, 303, 313 and 315, were propagated in PS80 and their host ranges and serology determined. The phages were serologically A. Their host ranges (Table 5) show that the first four phages resemble each other closely while phage 315/80 differs in its ability to attack strain 31A and, to a less extent, strain 42C. The host ranges of these phages differ from that of the A phage, 47C (Rountree, 1956). All five phages were easy to propagate and produced clear plaques on PS80, those of phage 684/80 being smaller than those of the other four. When they were applied undiluted to PS80, secondary resistant bacterial growth appeared in the areas of confluent lysis and was found to contain lysogenized cocci.

Table 5. *Host ranges of ‘converting’ phages of serological group* A

		Phage				
		B′b	684	308	313	315
Titre/ml. on propagating strain PS80		$6{\cdot}5 \times 10^7$	$1{\cdot}6 \times 10^9$	$1{\cdot}2 \times 10^{10}$	$3{\cdot}3 \times 10^9$	6×10^8
		Ratio: titre on PS80 to titre on indicator strain				
Indicator strain	6	10	10	16	18	20
	31/44	20	10	20	30	30
	31A	—	40,000	30,000	30,000	1·5
	42C	—	—	—	—	300
	42E	6,500	300	5,000	5,000	200
	47	—	300,000	10,000	10,000	100,000
	47C	3	8	6	10	10

The original lysogenic hosts of these phages and cultures of PS80 lysogenized with each phage were tested for cross-immunity to the phages. In these tests, the phages were applied undiluted and the results (Table 6) indicated reciprocal cross-immunity and hence identity of phages B′b and 308. Undiluted phage 684 produced ‘inhibition’ on all strains save its homologous strain and strain Bundaberg; these areas of inhibition in most cases contained scattered plaques. Similar appearances were observed with phage 313, which also gave ‘inhibition’ with its homologous strain 313 although not with strain Bundaberg. These reactions were considered to indicate that immunity to phages 684 and 313 was not absolute and also that the phage preparations probably contained mutant particles which were the cause of the scattered plaques. When the four phages were tested at higher dilutions, these reactions were not detectable and the phages would be regarded as identical. Phage

315/80 was clearly different from the other four phages. Strains 315 and PS80 (315) were identical and immune to all five phages, save for the minor reactions with phages 684 and 313. Nevertheless, all the other strains were fully sensitive to phage 315, thus providing an example of non-reciprocity in prophage immunity. Preliminary observations indicate that these phages are readily inducible by ultra-violet light.

Table 6. *Cross-immunity between 'converting' phages*

	Phage				
Strain	B′b/80	684/80	308/80	313/80	315/80
	Lytic reactions with undiluted phages				
B′b	–	–	–	–	(+ + + +)
684	–	–	–	( )	(+ + + +)
308	–	(+ +)	–	(+ + +)	+ + + +
313	–	(+ +)	–	( )	(+ + + +)
315	–	( )	–	+ +	–
PS80 (B′b)	–	(+ +)	–	(+ +)	+ + + +
PS80 (684)	–	(+ +)	–	(+ +)	(+ + + +)
PS80 (308)	–	+ +	–	(+ + +)	(+ + + +)
PS80 (313)	–	(+ +)	–	(+ +)	(+ + + +)
PS80 (315)	–	(+ +)	–	(+ +)	–

( ) = inhibition; (+ + + +) = confluent lysis with secondary growth; (+ +) and (+ + +) = inhibition with scattered plaques; + + + + = confluent lysis; – = no lysis.

Nature of the change in phage sensitivity of 80/81 *strains*

After lysogenization with the 'converting' phages, strains which originally typed as 80/81 became indistinguishable from the strains used as sources of the 'converting' phages. Two changes were involved: immunity to phage 47C; change from 'inhibition' with phages 52 and 52A to full susceptibility to attack by these phages. No clones lysogenic for these 'converting' phages were found which showed the 52/52A 'inhibition' pattern and it thus appeared that the phage sensitivity was closely linked with the presence of the A prophages.

Several hypotheses may be advanced to explain the mechanism of this change. The change is not one from complete resistance to full sensitivity but from 'inhibition' to full sensitivity. The 'converted' cocci behave as if some partial block in their ability to synthesize phages 52 and 52A has been removed. One hypothesis would accord a direct effect of the prophage on the behaviour of the lysogenized cocci. The mechanism of this change might be prophage substitution such as Gorrill (1957) found with the F phage 76, or the prophage might produce an alteration in the surface of cocci harbouring it so that they adsorbed the 52 and 52A phages more rapidly. An indirect effect is also possible in which the 'converting' phages act as the selecting agents for phage-sensitive mutants arising spontaneously in 80/81 cultures. If these phage-sensitive mutants were also the only cocci in 80/81 cultures capable of giving a lysogenic response when infected with the 'converting' phages, the observed result would be lysogenic cocci fully sensitive to phages 52 and 52A, but the mechanism of the change would be an indirect one. These hypotheses were examined experimentally.

Lysogenicity of 80/81 *strains*

PS80 was lysogenic and its phage was detectable on strain Bundaberg. Attempts to propagate this phage, PS80/B′b, met with considerable difficulties. It produced only minute fuzzy plaques and confluent lysis was never obtained on agar plates. Preparations from agar had titres of 10^5/ml. and were heavily contaminated with the lysogenic phage of strain Bundaberg. Attempts to produce high titres of PS80 by u.v. irradiation were equally unsuccessful. The prophage appeared to be non-inducible but, on one occasion, turbidity readings suggested that lysis of *c.* 10% of the cocci had occurred and a preparation was obtained with a plaque count on strain Bundaberg of 3×10^7/ml. This preparation was used to determine the host range and serology of the phage. This phage lysed only strain Bundaberg; it had no effect on any of the other strains containing 'converting' phages or on any of the propagating strains of the typing phages. Serologically, it was F.

The other 80/81 strains used for 'conversion' to 52/52A/80/81 were examined for the presence of this lysogenic F phage. It was found in strains 6351 and 6402, producing minute plaques on strain Bundaberg. It could not be demonstrated in broth cultures of PS81, 6352 or 9183. No other lysogenic phages were found in any of these 80/81 strains, although they were tested with all the propagating strains as indicators.

All the 'converted' cultures were tested for sensitivity to the F phage on the assumption that, if prophage substitution had occurred as the result of lysogenization, they should now be sensitive to the phage they had originally carried. All were resistant.

The F phage was sought in a culture of PS80 (308). Prophage 308 is inducible by u.v. irradiation. After irradiation, a lysate containing $2{\cdot}5 \times 10^9$ particles producing plaques on PS80/ml. was obtained which gave a plaque count on strain Bundaberg of $1{\cdot}5 \times 10^2$/ml. It was concluded that the F prophage was still present in PS80 cocci lysogenized with phage 308, although induction of prophage 308 in PS80 cocci had almost completely suppressed production of the non-inducible F phage. Taken as a whole, the results indicated that prophage substitution was not the mechanism of the change in phage sensitivity following lysogenization with the 'converting' phages.

Adsorption of phages 52 *and* 52A

If lysogenization with the 'converting' phage was correlated with a change in the phage receptors of the surface of the cocci, changes in the adsorption rates of phages 52 and 52A should be found. Adsorption experiments were set up, using these two phages mixed with cocci of PS80, cocci of PS80 (B′b) and PS80 (684) and, as controls, cocci of PS52 and PS52A. After growth for 4 hr. at 37° in glucose veal broth, volumes of 0·9 ml. of this culture were placed in tubes to which were added $CaCl_2$ to a final concentration of 300 μg./ml. and 0·1 ml. of phage suspension diluted to give 80–100 plaques when 0·01 ml. volumes of a broth control without cocci were plated. After 10 or 15 min. incubation in a water bath at 37°, the cocci + phage mixtures were centrifuged, the supernatant fluids removed and their content of unadsorbed phage determined by plating on PS 52 or PS 52A. The sedimented cocci were resuspended in 1 ml. volumes of broth and plated on PS 52 or 52A before the end of the latent period, in order to determine the proportion of infected cocci capable of producing phage 52 or phage 52A.

Table 7. *Adsorption and recovery of phage* 52 *from* PS 52 *cocci,* PS 80 *cocci and lysogenized* PS 80 *cocci*

Adsorption for 15 min. at 37°.

Cocci	Phage added (*a*)	Phage in supernatant (*b*)	Phage adsorbed (*a* − *b*)	Phage recovered in cocci	% of adsorbed phage recovered
PS 52	82	2	80	53	66
PS 80	82	1	81	2	2·5
PS 80 (B′b)	82	1	81	37	45·7
PS 80 (684)	82	1	81	30	37

Table 7 shows that there was no difference detected in the rate of adsorption of phage 52 to the four kinds of cocci tested, almost complete adsorption having occurred in 15 min. Recovery of phage 52 from its homologous cocci was not conspicuously efficient, 34 % of the adsorbed phage being lost. From cocci of PS 80, only 2·5 % of the adsorbed phage was recovered while the lysogenic 'converted' cocci yielded 45·7 and 37 % of the adsorbed phage. Similar results were obtained with phage 52A, 96–99 % adsorption occurring in 10 min.

It was concluded that lysogenization with the 'converting' phages was not associated with any alteration in the phage receptors on the surface of the cocci. Inability of the adsorbed phage to penetrate partially-resistant cocci was not excluded by these experiments. However, unpublished observations with another phage-staphylococcus system have shown that a large proportion of the cocci in 'inhibited' cultures are killed, indicating that the block in phage synthesis occurs after phage penetration of these partially resistant organisms.

Spontaneous change from 80/81 *to* 52/52A/80/81 *typing pattern*

The removal of the block in the ability to synthesize phages 52 and 52A might be due to a spontaneous mutation occurring independently of the presence of the 'converting' prophages. The occurrence of phage-sensitive mutants in cultures of PS 80 was investigated by a replica plating technique (Lederberg & Lederberg, 1952). Master plates were inoculated by spreading over their surfaces 0·01 ml. of a dilution of broth culture estimated to give 100–200 colonies. After incubation for 24 hr. at 37°, the plates were printed on to velvet which was used to inoculate two plates of the soft agar used for phage work; the first of these plates served as a control and the second had been spread with 0·1 ml. of a high-titre filtrate of phage 52. These plates were incubated at 30°. A trial with PS80 (B′b) cocci showed that all phage-sensitive clones could be recognized either by their small size on the phage-treated plates or by their absence from these plates. By this method, clones of PS80 fully sensitive to phages 52 and 52A were isolated. The site of the phage-sensitive colony having been ascertained by placing the phage-treated plate on top of the replicate without phage, the colony was picked from the plate without phage, grown in broth and tested for its phage reactions.

In four separate experiments (Table 8) 5 mutant colonies were detected among a total of 4375 colonies. Cultures made from these colonies typed as 52/52A/80/81 at the routine test dilution. Two were partially sensitive to phage 47C and three were resistant. In two experiments, no phage-sensitive colonies were detected among 3082 colonies. The master plates used for replication in these experiments were inoculated with cocci from 4 to 5 hr. broth cultures, while for those from which the phage-sensitive mutants were isolated 48 hr. cultures were used. This suggests that the organisms of PS80 have a fairly low mutation rate to phage sensitivity and that the mutants are more likely to be detected in older cultures where they have had the opportunity to accumulate. In one experiment, the back mutation to phage 52 'inhibition' was looked for in a culture made from one of these phage-sensitive clones but no partly resistant colonies were found among 1810 sensitives.

Table 8. *Occurrence in* PS80 *cultures of mutant clones sensitive to phages* 52 *and* 52A

Expt. no.	No. of plates	Total no. colonies	No. of mutant colonies
1	6	430	2
2	5	1016	—
3	8	1966	—
4	9	963	3

Taken as a whole, the results suggest that the 'conversion' to phage sensitivity in strains of 80/81 pattern may be the result of the selection of spontaneous mutants by the 'converting' phages. This hypothesis is supported by the fact that there is no apparent specificity among the lysogenizing phages, 'converted' lysogenic clones being found following the action of the F phage 863 as well as the various A phages. Demonstration of differences in the frequency of lysogenic responses in the original 80/81 cocci as compared with the mutant phage-sensitive cocci would be further evidence in favour of this hypothesis; such evidence is not yet available.

Changes in typing pattern in vivo

The demonstration that changes in phage typing pattern can occur *in vitro* either by spontaneous mutation or as a result of artificial lysogenization raises the question of whether such changes also occur *in vivo*. There seems no *a priori* reason why they should not occur, but their actual demonstration presents some difficulties. For example, when cultures typing as 80/81 and as

52/52A/80/81 are isolated from the same lesion it is impossible to exclude infection with two separate strains.

Examples of changes possibly due to spontaneous mutation have been found in our material. In one case of staphylococcal septicaemia, repeated blood cultures typed as 80/81 at the routine test dilution but on one occasion, a culture which was typed as 52/80/81 was isolated. Six single colonies of this culture were picked and typed; five were found to be 52/80/81 and the sixth 80/81.

A pair of cultures was received from Miss K. Key of the Commonwealth Serum Laboratories where they had been used in studies of staphylococcal pathogenicity in mice. The original strain 1406/1, typed as 80/81 with 47C/52/52A 'inhibition', while a subculture 1406/2 used in mouse challenge was 47C/52/52A/80/81. The origin of this fully sensitive strain was not known exactly, although it was presumed to have been derived from 1406/1. Neither strain was lysogenic.

One example was found of what appears to be change in typing pattern due to lysogenization. Two cultures were isolated from a patient, on successive days. The first strain (590) typed as 80/81 and was grown from an empyema. The second strain (594) was grown from sputum; it typed as 52/52A/80/81 and showed numerous phage plaques on the typing plates. From strain 594, two distinct phages were isolated, both serologically A, but differing in host range. Both these phages lysogenized strain 590 cocci and PS80 cocci, these cultures then typing as either 52/52A/80/81 or 47C/52/52A/80/81, depending on the phage used for lysogenization. The behaviour of these two phages will be discussed in detail elsewhere. The significance of the observation in the present connexion is that it affords suggestive evidence that the patient was originally infected with an 80/81 strain which later encountered a 'converting' phage in the respiratory tract.

DISCUSSION

Workers concerned with staphylococcal phage typing have generally assumed that some at least of the differences between strains belonging to the same phage group but which display different phage patterns are due to the presence in these strains of prophages which confer immunity to homologous and related phages. Lowbury & Hood (1953) lysogenized with homologous typing-phages thirteen of the strains used to propagate the typing phages and so produced strains immune to one or many phages depending on their original sensitivity. Examination of the data in their paper shows that resistance to a number of the phages occurred in blocks. Thus, lysogenization of PS47 with phage 47 produced a strain immune to phages 47B and 47C as well as phage 47; lysogenization of PS7 with phage 7 gave a strain immune to phages 7, 47, 47C, 47D, 75, 75A and 76. With the exception of phage 76 which is serologically F, all these phages are A. Similarly, lysogenization with B phages was followed by immunity to homologous and heterologous B phages.

Rountree (1956) pointed out that immunity following artificial lysogenization was serologically specific, in that lysogenization with an A phage conferred immunity to other A phages but not to B phages and vice versa. In the present paper, an example is given of this serological specificity of prophage immunity, but it is also shown that this is not a universal phenomenon since the prophage of an F phage immunized against an A phage. The reverse case of an A prophage immunizing against the F phage 76 is also implicit in Lowbury & Hood's data.

Of particular interest was the finding that, following lysogenization with certain A phages and an F phage, strains in which the multiplication of the B phages had been partially blocked became fully sensitive to these B phages. In these strains, the operational effect of lysogenization was not immunity but sensitivity. However, closer analysis of the phenomenon suggests that the mechanism of the change may be a subtle one, possibly involving selection of phage sensitive mutants able to give a lysogenic response to the 'converting' phages.

On the basis of the present results, it may be postulated that the basic pattern of phage sensitivity in this group of staphylococci is 47 C/52/52 A/80/81. This may be modified by lysogenization, by spontaneous mutation or by a combination of both mechanisms. Table 9 illustrates the various possibilities, all of which have been isolated as wild types or produced in the laboratory. Cocci of type 2 can be isolated from cultures of type 1 quite readily and the mutation of type 4 to type 1 cocci has been demonstrated by the replicate plating technique. The mutation of type 1 cocci to type 4 has not been demonstrated in the laboratory but that it may have occurred in nature and been the origin of the 80/81 strains now widely prevalent seems a reasonable hypothesis.

Table 9. *Various phage patterns within the phage group* 47 C, 52, 52 A, 80, 81 *and mechanism of their production*

Coccal type	Phage pattern at RTD*	Mechanism
1	47 C/52/52 A/80/81	Basic type; mutation of 4 to phage sensitivity
2	52/52 A/80/81	Mutation of 1 to phage 47 C 'inhibition'
3	52/52 A/80/81	Lysogenization of 1, 2, and 4 with A prophage
4	80/81	Mutation of 1 to 'inhibition' with phages 47 C, 52 and 52 A
5	47 C/52/52 A/80	Lysogenization of 1 with F prophage
6	52/52 A/80	Lysogenization of 2, 3 and 4 with F prophage
7	80	Lysogenization of 4 with F prophage
8	47 C/81	Lysogenization of 1 with B prophage
9	81	Lysogenization of 2, 3 and 4 with B prophage

RTD = routine test dilution.

The patterns of prophage immunity produced by lysogenization depends on the original phage sensitivity of the lysogenized cocci. Thus, 80/81 strains lysogenized by the F phage 863 became type 80, or, when the F phage is 'reduced' in phage-sensitive mutants of 80/81 cocci, 52/52 A/80, while strains of 47 C/52/52 A/80/81 pattern became 47 C/52/52 A/80. These results therefore give some information about the phage and prophage loci in the staphylococci. The loci 52, 52A and 80 appear to be closely linked but not identical. The loci for phage 47C on the one hand and for phages 81 and 863 on the other are not identical, while those for 81 and 863 are probably identical. It is also apparent that the A 'converting' phages although producing immunity to phage 47C are not identical with that phage.

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REFERENCES

Bynoe, E. T., Elder, R. H. & Comtois, R. D. (1956). Phage-typing and antibiotic-resistance of staphylococci isolated in a general hospital. *Canad. J. Microbiol.* **2**, 346.

Gorrill, R. H. (1957). Studies on lysogeny in staphylococci. *J. gen. Microbiol.* **17**, 254.

Gorrill, R. H. & Gray, R. A. (1956). The induction of bacteriophage in staphylococci. *J. gen. Microbiol.* **14**, 167.

Kellaway, C. H., McCallum, P. & Tebbutt, A. H. (1928). The fatalities at Bundaberg. Report of the Royal Commission. *Med. J. Aust.* ii, 2, 38.

Lederberg, J. & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.

Lowbury, E. J. L. & Hood, A. M. (1953). The acquired resistance of *Staphylococcus aureus* to bacteriophage. *J. gen. Microbiol.* **9**, 524.

Lwoff, A. (1953). Lysogeny. *Bact. Rev.* **17**, 269.

Rountree, P. M. (1949*a*). The phenomenon of lysogenicity in staphylococci. *J. gen. Microbiol.* **3**, 153.

Rountree, P. M. (1949*b*). The serological differentiation of staphylococcal bacteriophages. *J. gen. Microbiol.* **3**, 164.

Rountree, P. M. (1952). Serological studies of the multiplication of a staphylococcal bacteriophage. *Aust. J. exp. Biol. med. Sci.* **30**, 567.

Rountree, P. M. (1956). Variations in a related series of staphylococcal bacteriophages. *J. gen. Microbiol.* **15**, 266.

Rountree, P. M. & Freeman, B. M. (1955). Infections caused by a particular phage type of *Staphylococcus aureus*. *Med. J. Aust.* ii, 157.

Smith, H. W. (1948). Investigations on the typing of staphylococci by means of bacteriophage. II. The significance of lysogenic strains in staphylococcal type designation. *J. Hyg., Camb.* **46**, 82.

Williams, R. E. O. & Rippon, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.* **50**, 320.

Wilson, G. S. & Atkinson, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet*, i, 647.

(*Received* 1 *December* 1958)

Asheshov, E. H. & Rippon, J. E. (1959). *J. gen. Microbiol.* **20**, 634–643

Changes in Typing Pattern of Phage-Type 80 Staphylococci

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SUMMARY: Three temperate phages isolated from lysogenic staphylococci of phage type 52/52A/80 were used to lysogenize strains of phage-type 80. Two of these phages belonged to serological group A and one to serological group F. Lysogenization of a strain of phage-type 80 with one of the phages (287′) resulted in a change in the typing pattern from 80/81 to 52/52A/80. There was, therefore, a gain in sensitivity to phage 52 and 52A and a loss in sensitivity to phage 81. Lysogenization of type 80 strains with the second phage (581′) caused a loss in sensitivity to phage 81, while lysogenization with phage 7287′ caused a gain in sensitivity to phage 52 and 52A.

Loss in sensitivity to phage 81 after lysogenization with phage 287′ seems most probably due to specific prophage immunity since phages 287′ and 81 belong to the same serological group (A) and may be closely related. Loss in sensitivity to phage 81 after lysogenization with phage 581′ does not appear to be a case of specific prophage immunity since the two phages are serologically distinct and the phenomenon resembles examples of prophage interference observed in other organisms.

Rountree & Freeman (1955) described a number of hospital epidemics in Australia caused by a strain of staphylococcus which was almost completely resistant to the routine typing phages then in use. However, the two group-I phages 52 and 52A produced a few plaques when applied undiluted to a lawn of the organisms, and phage 52A was 'adapted' to this new type of staphylococcus. The adapted phage is now known as phage 80 and strains lysed specifically by this phage as phage-type 80. A second phage known as 81 was isolated independently in Canada (Bynoe, Elder & Comtois, 1956); it is capable of lysing the majority of phage-type 80 strains. Such strains are therefore more accurately described as phage-type 80/81. Since, however, phage 81 is not included in the set of 21 basic phages used in Great Britain, the strains are usually reported simply as phage-type 80. The 81 reaction will be referred to in this paper only when relevant.

Since the original description by Rountree & Freeman many epidemics caused by staphylococci of phage-type 80 have been described in different countries. During the past two or three years it has been noticed in this laboratory that, in some prolonged epidemics caused by phage-type 80, strains giving the typing pattern 52/52A/80 were also isolated, particularly during the later part of the epidemic. The 'classical' phage-type 80 coccus is lysed only by phage 80 at the routine test dilution (RTD), using the basic set of phages. Strains typing as 52/52A/80 therefore differ from phage-type 80 strains by two reactions and the usual practice in interpreting the results of typing patterns is to consider that strains differing from one another by two or more 'strong' reactions (at the RTD dilution) have a different origin. The frequency with which phage-type 52/52A/80 staphylococci occurred in conjunction with phage-type 80 strains, however, suggested that a close relationship existed between strains giving these two different typing patterns. Investigations were undertaken to see whether such a relationship did exist. It was decided first to see whether lysogenicity was, in any way, responsible for the two different typing patterns. It has been shown by a number of workers (Smith, 1948*a*, *b*; Lowbury & Hood, 1953; Rountree, 1956) that typing patterns of strains of staphylococci are often influenced by the carriage of a particular prophage. It has also been demonstrated in the phage-typing of *Salmonella typhi* that the presence of a 'type-determining' phage has a direct influence on the typing pattern of a strain (Anderson & Felix, 1953).

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RESULTS

Tests for lysogenicity

Staphylococcal strains of phage-types 80 and 52/52A/80 were examined for lysogenicity; 12 strains of phage-type 80 were examined and 39 strains of phage-type 52/52A/80. Cultures were grown in nutrient broth for 4–5 hr., centrifuged at 3000 rev./min. for 10 min. and the supernatant fluids spotted on staphylococcal strains of the opposite phage-type. None of the type-80 strains was lysogenic under these conditions; 33 out of the 39 strains of 52/52A/80 were lysogenic and carried phages capable of lysing and lysogenizing phage-type 80 strains.

Effect of lysogenization on typing patterns

Stocks of the phage carried by several strains of phage-type 52/52A/80 staphylococci were prepared by growing the lysogenic cultures in broth for 4–5 hr., centrifuging down the cocci, and filtering the supernatant fluid through sintered-glass filters (APD 0·2 μ) or membrane filters (APD 0·25 μ). The filtrates were tested for sterility and were then used to lysogenize a number of typical phage-type 80 strains. The filtrate was spotted undiluted on to a lawn of the staphylococci on nutrient agar. Secondary growth in the areas of lysis was visible on the following day and this was picked and streaked on nutrient agar plates to give isolated colonies. Several well-isolated colonies were picked into broth, grown for 4–5 hr. and tested for lysogenicity. They were considered to be lysogenic when (1) the supernatant fluid of a centrifuged sample was shown to contain the particular phage used to lysogenize them and (2) the culture had changed from sensitivity to resistance to this phage (i.e. had become immune). When it was established that the cultures were lysogenic, their typing pattern was tested by using the 21 basic phages in the routine typing set and the 21 extra phages used in this laboratory to type strains which are non-typable with the basic set.

It was possible to distinguish three distinctly different phages carried by the various strains of phage-type 52/52A/80. These were: phage 287′ isolated from strain 287; phage 581′ isolated from strain 581; phage 7287′ isolated from strain 7287. There are probably others, but these three phages were examined in some detail; their characteristics are summarized in Table 1 which shows only the reactions with four of the typing phages since the strains were resistant to all the other typing phages. These four phages were three of the group I typing-phages (52, 52A and 80) and phage 81. All of the phage-type 80 staphylococci used in these experiments were lysed equally well by phage 81 at the routine testing dilution and were therefore of phage-type 80/81 before lysogenization. In general our results agree with those reported by Rountree: our phage 581′ resembles her phage 863 and our phage 7287′ resembles her serological group A phages. Lysogenization with each of the three phages resulted in a change in the typing pattern of the strain lysogenized which took the form of a gain in sensitivity or a loss in sensitivity or a concurrent gain in sensitivity to two phages and a loss to a third. In general the typing pattern resulting from lysogenization corresponded with that of the strain from which the phage was derived.

Table 1. *Characteristics of three temperate phages derived from lysogenic strains of phage-type* 52/52A/80

The typing patterns recorded below were those using the phages at the routine testing dilution. Degrees of reaction recorded as follows: ++ = 50 or more plaques; + = 20–50 plaques; ± = less than 20 plaques.

		Typing pattern of strain from which phage was derived				Typing pattern of 80/81 strains after lysogenization				Changes in phage-sensitivity following lysogenization	
Phage	Sero-logical group	52	52A	80	81	52	52A	80	81	Gains	Losses
287′	A	+	±	++	−	+	±	++	−	52, 52A	81
581′	F	+	+	++	−	−*	−*	++	−	−*	81
7287′	A	++	++	++	++	++	++	++	++	52, 52A	—

* The majority of colonies of phage-type 80/81 strains lysogenized with phage 581′ were sensitive only to phage 80. However, occasional colonies showed sensitivity to phages 52 and 52A as well.

The three phages were distinguished most easily on the basis of the resistance patterns they conferred against one another when a single strain of phage-type 80 was lysogenized separately with each of the three phages (see Table 2). The convention used in designating these indicator strains was to put the number of the carried phage in parentheses after its strain. Thus, strain 6346 (287′) refers to staphylococcal strain 6346 lysogenized with phage 287′. This set of indicator strains was used to characterize the phages carried by other lysogenic strains of phage-type 52/52A/80. Of the 33 lysogenic staphylococcal strains tested 15 carried a phage corresponding to phage 287′, 2 carried a phage like 581′, and 6 a phage like 7287′. Ten strains carried a phage or phages able to lyse all 4 indicator strains. These differed from any of the three main phages but, as they were not further investigated, it is not known whether they represented 'converting' phages, i.e. phages capable of changing the typing pattern. In addition, 6 strains of phage-type 80 which were resistant to phage 81 were examined for lysogenicity. All 6 were lysogenic and carried a phage whose lytic spectrum corresponded to that of phage 581′.

The results suggest that the change in typing pattern occurred as the direct result of lysogenization. The loss in sensitivity to phage 81 after lysogenization with phage 287′ may be due to prophage immunity, since both phage 287′ and phage 81 belong to the same serological group (A) and may be closely related. Moreover, strains of phage-type 80 lysogenized with phage 581′ lost sensitivity to both phage 81 and to phage 287′ (Tables 1 and 2).

Table 2. *Lytic action of the three phages on a set of indicator strains obtained by lysogenizing a single strain,* 6346, *of phage-type* 80, *with each of the three phages separately*

Indicator strains	Phages 287′	581′	7287′
6346	+	+	+
6346 (287′)	−	+	+
6346 (581′)	−	−	+
6346 (7287′)	+	+	−

Loss of sensitivity to phage 81 after lysogenization with phage 581′ may well be due to prophage interference; it does not appear to be due to specific prophage immunity since the two phages are serologically distinct. A similar phenomenon occurs in *Salmonella typhi*, where lysogenization with a 'type-determining' O phage renders the strain resistant to the unrelated *Vi* typing phage (Anderson & Felix, 1953). It has also been described for the three temperate phages, λ, P1 and P2 (Lederberg, 1957; Bertani, 1958).

Gain in sensitivity to phages 52 and 52A after lysogenization with phages 287′ and 7287′ (and occasionally with phage 581′) is more difficult to explain. However, it should be noted that staphylococci of phage-type 80 are not completely resistant to phages 52 and 52A; they are capable of adsorbing the phage particles at an appreciable rate. Adsorption of a single phage particle appears to 'kill' a coccus in that this is lost as a colony-former. It would appear therefore that phage 52 and phage 52A are able to penetrate into cells of phage-type 80 but that their synthesis is blocked at some later stage. It is also worth noting that the three group I phages, 52, 52A and 80 are phenotypic modifications of a single phage-type and the difference between strains able to synthesize one or all three modifications may be very slight (unpublished observations).

There appeared to be four possible explanations of the gain in sensitivity to phages 52 and 52A after lysogenization. These are: (1) lysogenic conversion; (2) selection of pre-existent mutants; (3) transduction; (4) prophage substitution. Several experiments to test the validity of these various possibilities were carried out. All of these experiments were done with staphylococci of strain 6346, phage-type 80 and phage 7287′.

Lysogenic conversion would appear to be the simplest explanation of the gain in sensitivity to phages 52 and 52A in lysogenized phage-type 80 cells. It assumes that the presence of the prophage is necessary to overcome the block in the synthesis of the two phages noted above, in the same way that the presence of the prophage is necessary to convert non-toxigenic strains of *Corynebacterium diphtheriae* to toxin-producing strains (Freeman, 1951). The simplest way to test this explanation appeared to be to 'cure' the lysogenic strain and see whether it would revert to phage-type 80. The method used to isolate non-lysogenic mutants in the lysogenic culture was that described by Lederberg & Lederberg (1953). Two different lysogenic strains were included in these experiments. Strain 7287 of phage-type 52/52A/80 from which phage 7287′ was isolated; strain 6346 (7287′), i.e. strain 6346 of phage-type 80, lysogenized with phage 7287′ and thus converted to phage-type 52/52A/80. Broth cultures of both strains were used to flood nutrient agar plates containing 2×10^{-2} M-sodium citrate. The plates were u.v. irradiated sufficiently to eliminate all but 100–200 colonies/plate (about 1 in 10^6 survival). These colonies were then replicated on plates flooded with a suitable indicator strain (6346). This made it possible to distinguish between lysogenic and non-lysogenic colonies, because the former were always surrounded by a narrow zone of phage lysis. Non-lysogenic mutants were isolated from each of the two staphylococcal strains; both proved to be of phage-type 52/52A/80. The continued presence of the prophage 7287′ was therefore not necessary to maintain the change of phage-type 80 strains to phage-type 52/52A/80, and the observed change in typing pattern could not be due to simple lysogenic conversion.

Selection of pre-existing mutants. This explanation was first proposed by Rountree (personal communication, and 1959). It supposes that the converting phage acts as a selector of pre-existent mutants. Rountree found that old cultures of phage-type 80/81 staphylococcal strains contained mutants of phage-type 52/52A/80/81 which she was able to isolate by a replica plating technique. The mutants were present in the proportion of *c.* 1/200 cocci in 24 hr. broth cultures. She suggested that the action of the 'converting' phages was a direct selective one—i.e. the phages lysed the parent 80/81 cocci and lysogenized the mutant 52/52A/80/81 cocci. The mutants therefore would be the only cocci to survive exposure to the phage and would survive by virtue of their being lysogenized. We attempted to isolate these mutants from strain 6346 by using the replica plating method, but failed. A total of 5774 colonies was examined in five different experiments, the highest number in any one experiment being 1465. We used 4–5 hr. broth cultures of strain 6346, and it is quite possible that the mutants were not present at that time or were not present in sufficient concentration to be detected by the screening method. It seemed, however, that we had an example of the mutant in the culture of strain 6346 which was first lysogenized with phage 7287′ and subsequently de-lysogenized (see above); this strain is referred to as 6346DL. Experiments were carried out to compare the frequency of lysogenization of strain 6346 with that of strain 6346DL, using phage 7287′. If the selection explanation were correct the frequency of lysogenization of strain 6346 DL should be much higher than that of strain 6346.

The experiments were carried out as follows. The two strains were grown in broth for 4 hr. to *c.* 5×10^7 colony-formers/ml. Each culture was then divided into two parts. Phage 7287′ was added to one part to give 1×10^9 phage particles/ml., so that there were about 20 phage particles/colony-former; this is referred to as the adsorption mixture. The other half of the culture served as a control of the initial concentration of bacteria (bacterial control) and was examined for the presence of mutants of phage-type 52/52A/80. The adsorption mixtures were shaken at 37° for 10 min. to allow phage adsorption and were then diluted 1/10 with broth containing sufficient antiserum to inactivate 99·5 % of unadsorbed phage in 5 min. Five minutes were allowed for serum inactivation and the tubes were further diluted to contain 100–200 colony formers/0·1 ml.; this volume was then spread on each of several plates. The same dilution was made from the bacterial control tubes and similarly plated. After overnight incubation counts were made of the colonies produced from the bacterial control tubes and from the adsorption mixtures. The survivors from the adsorption mixtures were replicated on plates flooded with staphylococcal strain 6346, in order to detect lysogenic colonies amongst the survivors. Colonies corresponding to those which appeared to be lysogenic were then picked from the master plates and tested for lysogenicity and sensitivity to phages 52, 52A and 80. Colonies on the plates from the bacterial control tubes were replicated on plates flooded with phage 52, in order to detect any mutants of phage-type 52/52A/80.

In each of three experiments with strain 6346 we were unable to detect any mutants of phage-type 52/52A/80 in the bacterial control. All of the colonies from the bacterial control of strain 6346 DL were sensitive to phage 52 when tested by the replica-plate method. This was the expected result and was included as a control on the method of testing for phage 52-sensitive colonies. The results of the platings from the two adsorption mixtures (6346 and 6346 DL) are given in Table 3. In analysing the results we have listed four possible responses: lytic, lysogenic, mixed, non-lysogenic. The % cocci which gave a lytic response was calculated by subtracting the number of survivors from the initial concentrations of cocci, represented by the bacterial control. Amongst the survivors, colonies were considered to be lysogenic when they were shown to be resistant to phage 7287′, to carry phage 7287′ and to be stable on subculture. All the lysogenic colonies were found to be of phage-

Table 3. *Frequency of lysogenization of strains 6346 and 6346 DL by phage 7287′*

Staphylococcal strain	Type of response following 10 min. exposure to phage				Frequency of lysogenization
	Lytic (*a*)	Lysogenic (*b*)	Mixed (*c*)	Non-lysogenic (*d*)	$\frac{(b)}{(a)+(b)+(c)}$
6346	87 %	1·57 %	8·8 %	2·61 %	0·016
6346 DL	88·5 %	2·05 %	8·2 %	1·31 %	0·020

type 52/52A/80. A large proportion of the survivors were classified as 'mixed'; these were unstable and tended to undergo spontaneous lysis on subculture. As a rule they were partially resistant to phage 7287′; they were shown to be carrying phage 7287′ but appeared to consist of a mixture of phage-sensitive and lysogenic phage-resistant cocci. They probably arose originally from clumps of cocci of which only a proportion became lysogenized during the 10 min. adsorption period, the rest remaining non-lysogenic and phage-sensitive. Non-lysogenic colonies were tested in a few instances and proved to be fully sensitive to phage 7287′; these colonies presumably arose from cocci which had not become infected during the adsorption period. The results of these experiments did not support the selection explanation, for two reasons.

(*a*) We were unable to detect the mutants of phage-type 52/52A/80 in the bacterial control suspension of strain 6346, although 1·57 % of the cocci gave a stable lysogenic response when exposed to phage 7287′ and were phage-type 52/52A/80. However, in interpreting the results one fact needs to be considered. Only a small proportion of the colonies in the experiments arose from single cocci. Smears made from the suspensions of cocci immediately before beginning the experiment and examined microscopically showed that the average unit consisted of between 2·5 and 3·0 cocci. This undoubtedly explains why so many of the survivors from the adsorption mixtures gave mixed responses. It is probable that this fact biased the results against support of the selection explanation. If a mutant is present as one coccus in a clump of 4 cocci, the colony which develops from this clump will contain only 25 % of cocci susceptible to phage 52 (assuming identical growth rates for the mutant and the wild-type cocci). Whether such a colony would be recognizable as sensitive to phage 52 by the replica-plating method seems doubtful. If the same clump of cocci were exposed to phage 7287′ and only the mutant became lysogenized, the colony that formed would be classified as giving a lysogenic or a mixed response, depending on how soon the other, phage-sensitive, non-lysogenic cocci were eliminated by lysis with phage 7287′.

(*b*) Comparison of the frequency of lysogenization of strain 6346 and 6346DL failed to show any significant differences although, on the basis of our second explanation (selection of pre-existing mutants) strain 6346DL should have shown a much higher frequency than strain 6346. Our general conclusion is therefore that this explanation is not tenable.

Transduction. The assumption that the change in typing pattern was due to transduction in a manner similar to that described for other organisms was eliminated for two reasons: (i) transduction would need to be extremely efficient, since all of the lysogenized colonies tested were found to have the new typing pattern; (ii) the character of the strain on which the 'converting' phage was last propagated did not affect the change; phage 7287′ propagated on strain 6346 (phage-type 80) gave the same results as phage 7287′ derived by spontaneous lysis of strain 7287 (phage-type 52/52A/80).

Prophage substitution. The fourth possibility was that cocci of phage-type 80 were originally lysogenic and that lysogenization with a 'converting' phage caused prophage substitution with the loss of the original prophage. Assuming that the presence of the original prophage inhibited synthesis of phage 52 and 52A, substitution with another phage might have the effect of overcoming this inhibition and of allowing synthesis to occur. In all our experiments the different strains of phage-type 80 were tested for lysogenicity by using strains of the opposite phage-type (52/52A/80) as indicator strains; in no instance was any phage detected. This failure may have been due to the unsuitability of the indicator strains. Further tests were made by using a single strain of phage-type 80 (strain 6346) as source of the hypothetical phage and using as indicators strains of 6364 lysogenized separately with each of the three 'converting' phages. If prophage substitution had occurred these lysogenized strains should have been sensitive to the phage that was lost and have served as suitable indicators for such a phage. No phage was detected. Filtrates of strain 6346 were then tested on two strains of staphylococci known to be sensitive to the majority of staphylococcus phages; but again no phage was detected. The experiments described by Rountree (1959) indicated that prophage substitution was not responsible for the change in typing patterns. It is, of course, possible that strains of phage-type 80 are really lysogenic but produce only 'defective' phage which cannot mature to produce lysis. There is no evidence for this at the present.

DISCUSSION

The present results show that the typing pattern of phage-type 80 strains of staphylococci can be changed as the result of lysogenization *in vitro* with specific temperate phages derived from strains of phage type 52/52A/80. With three different phages, three different patterns were obtained. With two of the three phages lysogenization caused a loss in sensitivity to phage 81, and with two of the phages there was consistently a gain in sensitivity to phages 52 and 52A. No experiments were carried out to explain the loss in sensitivity to phage 81, but we consider that in one case the effect was probably due to specific prophage immunity and in the other case to prophage interference.

Gain in sensitivity to phage following lysogenization is a more unusual observation, although not unique. Uetake, Luria & Burrous (1958) reported that strains of *Salmonella anatum* became sensitive to phage E^{34}, to which they had hitherto been resistant, as the result of lysogenization with phage E^{15}; they showed that this gain was due to production of a new somatic antigen which was the receptor for phage E^{34}. Brandis & Storch (1957) reported a gain in sensitivity to phage 3a and 3aI in strains of *S. paratyphi* B after lysogenization with a temperate phage (St). The phenomenon was due to recombination between phage St and the typing phage 3a and 3aI respectively.

The gain in sensitivity to phage 52 and 52A reported here is not due to a change in the phage receptor sites on the cell wall of the staphylococci used, since phage-type 80 cocci absorbed both phages at a measurable rate. The block in the synthesis of the two phages appears to occur at a later stage, after adsorption and probably after penetration, because there is evidence that adsorption of either phage killed phage-type 80 cocci. Four explanations were put forward to explain the phenomenon (transduction, lysogenic conversion, selection of pre-existent mutants, prophage substitution) but the results failed to support any of these. Transduction appeared unlikely because of the high efficiency of the change, and was excluded when it was found that the ability of the phage to produce the change was independent of the character of the last strain used to propagate the phage. Selection of pre-existent mutants appeared probable, since Rountree had shown that mutants of phage-type 52/52A/80 existed in old cultures of phage-type 80 strains. However, studies of frequency of lysogenization in other systems showed that, at least, in these other systems, the frequency of lysogenization depended on the physiological state of the host organisms and could be altered by altering the external conditions (Lieb, 1953; Bertani & Nice, 1954). The few experiments we made with one staphylococcal strain of phage-type 80 and one of the three 'converting' phages suggested that the frequency of lysogenization was too high to be accounted for by selection of pre-existent mutants.

We were unable to prove or disprove the occurrence of prophage substitution, since no active phage could be demonstrated in filtrates of cultures of phage-type 80 cocci. Our failure may have been due to lack of a suitable indicator strain or because phage-type 80 cocci produced only 'defective' phage. That phage-type 80 cocci may contain an active phage is shown by an observation of Rountree (1959) who found that one staphylococcal strain of phage-type 80 (the propagating strain for phage 80) carried a phage of serological group F which was released both by the original strain and by the strain after lysogenization with one of the 'converting' phages. All that we can say is that there is no evidence so far to support the explanation of prophage substitution.

Lysogenic conversion, as generally understood, cannot explain the results, because the change in typing pattern remained after the loss of the 'converting' phage. In other cases of lysogenic conversion, loss of prophage has resulted in a reversion to the pre-lysogenic character (e.g. Groman, 1955). However, in the absence of any other explanation and because of the available experimental evidence, we suggest that the change in typing pattern occurs as the direct result of lysogenization, but that once this change has occurred it remains and is independent of the continuing presence of the prophage.

The work reported here originated in the observation that staphylococcal strains of typing pattern 52/52A/80 seemed to appear with undue frequency in epidemics due primarily to type-80 strains. We have since seen six cases in which staphylococci with both typing patterns were isolated from the same person. Bearing in mind the great number of phage patterns among staphylococci, this is strong evidence that the change from the 80 to the 52/52A/80 pattern, or the reverse, can occur in nature. In fact, the epidemiological evidence suggests that the change is usually from 80 to 52/52A/80. But further investigation is needed on this point because strains of type 80 are undoubtedly much more often the cause of epidemics of hospital infection than are strains with the pattern 52/52A/80.

REFERENCES

ANDERSON, E. S. & FELIX, A. (1953). The *Vi*-type determining phages carried by *Salmonella typhi*. *J. gen. Microbiol.* **9**, 65.

BERTANI, G. (1958). Lysogeny. *Advanc. Virus Res.* **5**, 151.

BERTANI, G. & NICE, S. J. (1954). Studies on lysogenesis. II. The effect of temperature on the lysogenization of *Shigella dysenteriae* with phage P1. *J. Bact.* **67**, 202.

BRANDIS, H. & STORCH, I. (1957). Über eine Kreuzung von Paratyphus B-Bakteriophagen. *Z. Hyg. InfektKr.* **144**, 164.

BYNOE, E. T., ELDER, R. H. & COMTOIS, R. D. (1956). Phage-typing and antibiotic-resistance of staphylococci isolated in a general hospital. *Canad. J. Microbiol.* **14**, 167.

FREEMAN, V. J. (1951). Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. *J. Bact.* **61**, 675.

GROMAN, N. (1955). Evidence for the active role of bacteriophage in the conversion of non-toxigenic *Corynebacterium diphtheriae* to toxin production. *J. Bact.* **69**, 9.

LEDERBERG, E. M. & LEDERBERG, J. (1953). Genetic studies of lysogenicity in *Escherichia coli*. *Genetics*, **38**, 51.

LEDERBERG, S. (1957). Suppression of multiplication of heterologous bacteriophages in lysogenic bacteria. *Virology*, **3**, 496.

LIEB, M. (1953). The establishment of lysogenicity in *Escherichia coli*. *J. Bact.* **65**, 642.

LOWBURY, E. J. L. & HOOD, A. M. (1953). The acquired resistance of *Staphylococcus aureus* to bacteriophage. *J. gen. Microbiol.* **9**, 524.

ROUNTREE, P. M. & FREEMAN, B. M. (1955). Infections caused by a particular type of *Staphylococcus aureus*. *Med. J. Aust.* ii, 157.

ROUNTREE, P. M. (1956). Variations in a related series of staphylococcal bacteriophages. *J. gen. Microbiol.* **15**, 266.

ROUNTREE, P. M. (1959). Changes in the phage-typing patterns of staphylococci following lysogenization. *J. gen. Microbiol.* **20**, 620.

SMITH, H. W. (1948*a*). Investigations on the typing of staphylococci by means of bacteriophage. I. The origin and nature of lysogenic strains. *J. Hyg., Camb.* **46**, 74.

SMITH, H. W. (1948*b*). Investigations on the typing of staphylococci by means of bacteriophage. II. The significance of lysogenic strains in staphylococcal type designation. *J. Hyg., Camb.* **46**, 82.

UETAKE, H., LURIA, S. E. & BURROUS, J. W. (1958). Conversion of somatic antigens in Salmonella by phage infection leading to lysis or lysogeny. *Virology*, **5**, 68.

(*Received* 1 *December* 1958)

Ryan, F. J. & Kiritani, K. (1959). *J. gen. Microbiol.* **20**, 644–653

Effect of Temperature on Natural Mutation in *Escherichia coli*

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SUMMARY: The mutation rate from histidine auxotrophy to prototrophy in *Escherichia coli* was measured during growth and in the stationary phase at different temperatures. During growth the rate of mutation/mutable unit/hr. had the same temperature coefficient as the rate of growth. Therefore the rate of mutation/mutable unit/generation was the same at all temperatures. The temperature coefficient of the rate of mutation/unit/hr. during the stationary phase may be the same as that during growth, indicating that although mutation is slower in this phase it may involve the same process as during growth.

In *Serratia marcescens* the percentage of colour mutants in colonies of different size but of the same age has been shown to be equal (Kaplan, 1947). Similarly, it was shown by Novick & Szilard that mutations of resistance to bacteriophage T_5 may occur in *Escherichia coli* at a rate which is a function of absolute time and, within limits, not of the number of generations (Novick & Szilard, 1950, 1953; see also Novick, 1956; Lee, 1953; Fox, 1955; Moser, 1958). The rate of mutation in both of these cases was further shown to have a Q_{10} of *c.* 2. Because Novick & Szilard used the chemostat which allowed the maintenance of identical growth rates at different temperatures, they were able to separate experimentally the effect of temperature on mutation from its effect on growth, a condition not met in previous studies on temperature and mutation. Unfortunately the chemostat device employed, which provides the desirable conditions of continuous logarithmic growth in a virtually constant environment, can be used simply for the measurement of few mutations, so far as is known; many mutants do not increase in the linear way predicted by theory, perhaps because of selection (Novick & Szilard, 1950; de Rothschild, 1954; Moser, 1958). Consequently, the generality of Novick & Szilard's conclusions on the properties of mutations in bacteria may be questioned.

Stocker (1949) found that the rate of mutation of factors influencing flagellar antigens and the growth of *Salmonella typhimurium* itself were influenced in the same way by temperature and nutritional conditions. Witkin (1953), using a completely different technique and resistance to bacteriophage T_1 in *Escherichia coli* showed that the temperature coefficients of spontaneous mutation/unit time and of cell division were identical; the Q_{10} again was *c.* 2. Thus, the natural mutation rate/generation was constant over a wide range of temperatures, causing different rates of growth. Furthermore, Fox (1955) and Moser (1958) showed that in the chemostat at rapid rates of growth (approaching those of unrestricted growth at 37°) the rate of mutation of *E. coli* to resistance to phage T_5 was a function of generation time.

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The mutation from histidine auxotrophy (h^-) to prototrophy (h^+) in *Escherichia coli* offers several advantages in this connexion. It cannot be investigated in the chemostat, probably for reasons of selection resulting at least in part from the fact that new mutants do not increase in number until they have become homocaryotic through division (Ryan & Wainwright, 1954; de Rothschild & Ryan, 1953). The basis of this segregation delay resides in the fact that h^+ is a dominant condition. Recessive mutations such as those to phage resistance, when studied by the method employed by Witkin, show a long delay in appearance. This interesting phenomenon is not completely understood (Witkin, 1956) but it is known that it is affected by temperature. On the other hand, a dominant prototrophic mutant, such as h^+, must express its character on minimal medium before it can divide. The $h^- \rightarrow h^+$ mutation can be measured among bacteria which are in a stationary phase where they do not divide (Ryan, 1955). Thus the responses of growth, of multiplication and of mutation to temperature can be separated. The present paper reports the effect of temperature on mutation $h^- \rightarrow h^+$ during growth and in the stationary phase.

METHODS

The basic techniques used were the usual ones employed in our investigations of mutation from the histidineless (h^-) to the histidine independent (h^+) condition in strain 15 of *Escherichia coli* (Ryan, 1955). Medium supplemented with enough histidine to support the growth of *c.* 10^7 h^- organisms/ml., was distributed in 2 ml. volumes into large numbers of small test tubes, and were freshly inoculated to contain *c.* 25 washed h^- bacteria/ml. The cultures were placed in incubators maintaining the specified temperatures to within 1°.

Viable counts were made at intervals by plating appropriate dilutions from a sample of these tubes on minimal agar supplemented with 25 μg. L-histidine HCl/ml. and incubating at 37°. In this way the rate of growth, the total yield of bacteria and the rate of death during the stationary phase was determined. Until the stationary phase had set in, the number of viable bacteria was indistinguishable from the total number measured in a counting chamber. Thereafter, although the total number remained constant for weeks, the viable number decreased according to the function

$$N_t = N_0 e^{-kt}, \tag{1}$$

where N_t is the number of viable bacteria at time t, N_0 the number at time O and k a constant. During growth, mutations from h^- to h^+ occurred in some of the tubes. The h^+ mutants are not limited by the exhaustion of histidine but overgrow the cultures in which they are found until they use up the glucose present which allows the growth of about 5×10^8 bacteria/ml. Thus, cultures with adaptive h^+ overgrowths were turbid and those without h^+ mutants appeared clear to the naked eye. Among groups of 100 tubes reserved for this purpose, the frequency of tubes without mutants (P_0) can be determined. By using the zero term of the Poisson formulation

$$P_0 = e^{-m}, \tag{2}$$

m, the average number of mutations, can be calculated. This can be inserted, along with N, the average number of bacteria/tube at the end of growth, into the equation which defines the mutation rate:

$$a = \frac{m \ln 2}{N}, \tag{3}$$

where a is the chance of mutation/bacterium/generation.

The h^+ adaptive overgrowths did not cease to appear at the time expected when the h^+ mutants arose during growth; rather new adaptations occurred for weeks after the onset of the stationary phase. In order to determine the length of the interval required for the overgrowth by h^+ mutants which arose at the very end of growth, single h^+ mutants marked with an inability to ferment lactose were added at that time (average addition 0·5 h^+ lac^- bacteria/culture; therefore, 30 % of the cultures should have received one, 8 % two, and 1 % three bacteria). When the cultures had adapted they were streaked on lactose eosin methylene-blue agar to determine whether the adaptation was due to an added lac^- bacterium. Since lac^- and lac^+ bacteria grow on glucose at equal rates the average times for overgrowth were determined at the different temperatures. These times, as well as those required for the h^- bacteria to reach the stationary phase, are shown in Table 1. The sum of these times is that after which the tubes were observed in order to estimate the frequency of mutations arising during growth.

Adaptations which occurred after this time were counted for the measurement of mutations occurring during the stationary phase (Ryan, 1955). The rate/bacterium/hr. ($\mu.$) was calculated by the formula

$$\mu. = \frac{km}{N_0\,(1-e^{-kt})}, \tag{4}$$

where k is taken from equation (1), m is calculated according to equation (2) for adaptations occurring after the total times in Table 1, t is the length of the interval in hours (hr.) over which observations were made and N_0 is the number of bacteria present at the beginning of the interval minus the number of hours required for adaptive overgrowth at the various temperatures. It has already been shown that these adaptations in the stationary phase are the result of mutations which occur during that period in non-dividing organisms (Ryan, 1955).

RESULTS

Table 2 shows the way in which the rate of growth and the rate of mutation during growth varied with temperature. Over the range of temperatures from 20 to 30° the rates of mutation calculated/organism/generation were indistinguishable. Because generations require longer times as the temperature decreases, the rate of mutation/hr. decreased with temperature.

The measurements at 15° require special consideration. It is possible that for technical reasons the total number of organisms present was not accurately estimated. But even when the number of organisms actually present is taken as $1{\cdot}7 \times 10^7$ (the average of experiments at other temperatures), the calculated rate of mutation is still significantly higher because of the absolutely greater average number of mutations/culture. It was thought that at this temperature the average number of mutable units/organism might be larger, although the rate of mutation/mutable unit/generation might be the same as at other temperatures. Previous work at 37° had shown that the number of mutable units was *c.* 4 (Ryan & Wainwright, 1954; Ryan, Fried & Schwartz, 1954). Serial transfer experiments at 15° were therefore performed to measure the rate of increase of the h^+ mutants. The mutation rate calculated from the slope of the increase curve should be equal to the rate calculated by the Poisson method if there is one mutable unit/organism, reduced to 1/2 if there are 2 mutable units, to 1/4 if there are four, and so on. In the serial transfer

Table 1. *Times for the appearance of* h^+ *mutants of* Escherichia coli *at different temperatures*

Temp.	Time to the stationary phase in limiting histidine (hr.)	Time for overgrowth of single h^+ lac^- bacteria (hr.)	Time at which mutants arising during growth should be observed as adaptive overgrowths (hr.)
15°	132	160	292
20°	64	83	147
25°	44	48	92
30°	34	35	69
37°	22	27	49

Table 2. *The rates of growth and of mutation during growth at different temperatures*

Temp.	No. of experiments	Generation time (hr.)	Average no. of viable bacteria/culture ($\times 10^{-7}$)	Average no. of mutations/culture	Mutation rate/bacterium/generation ($\times 10^{-8}$)	Range ($\times 10^{-8}$)	Mutation rate/bacterium/hr. ($\times 10^{-8}$)	Range ($\times 10^{-8}$)
15°	7	4·9	1·1	1·14	7·0 (3·5)*	5·1–9·3	1·4 (0·7)*	1·0–1·9
20°	5	2·5	1·6	0·67	3·0	2·5–3·5	1·2	1·0–1·4
25°	6	1·7	1·8	0·71	2·9	2·0–3·8	1·7	1·2–2·3
30°	7	1·3	1·6	0·59	2·9	1·9–5·3	2·2	1·5–4·1
37°	5	0·92	1·8	0·83	3·2	2·7–3·6	3·5	3·0–3·9

* If adjusted for the number of mutable units present; see text.

experiments the proportion of h^+ mutants increased exponentially rather than linearly according to the relation

$$\frac{h^+}{h^-} = \frac{ga}{n} \tag{5}$$

where g is the number of generations, a the rate of mutation/organism/generation and n the average number of mutable units/organism. The exponential results obtained indicated that there was a selection for the h^+ bacteria at 15°, a phenomenon not observed at 37°. The initial slope of the increase curve was, however, *c.* 9×10^{-9} which when compared with the value of 7×10^{-8} found in the Poisson experiments, suggested the presence of 8 instead of 4 mutable units as had been found at 37° by the same scheme of comparison (Ryan & Wainwright, 1954). Therefore, when the mutation rates at other temperatures were divided by 4 and that at 15° by 8, indistinguishable mutation rates/mutable unit/generation are obtained.

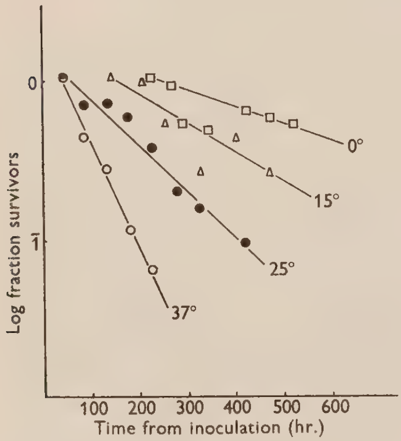

Fig. 1

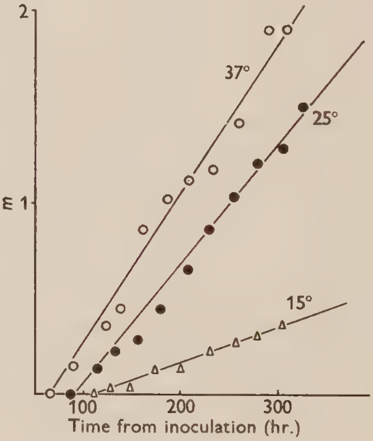

Fig. 2

Fig. 1. Examples of decrease in viability during the stationary phase at different temperatures. The rate constants from equation (1) were: for 0°, $2{\cdot}5 \times 10^{-3}$; for 15°, $4{\cdot}2 \times 10^{-3}$; for 25°, $6{\cdot}4 \times 10^{-3}$; for 37°, $1{\cdot}5 \times 10^{-2}$.

Fig. 2. Examples of increase in the average number of mutations/culture, μ, during the stationary phase at different temperatures. Equation (2) was used to calculate m; P_0 was set at 1·00 after the mutants arising during growth had been accounted for at the times indicated in Table 1. The abscissa for the curve for 15° has been shortened by 180 hr. for convenience. The data at 15° yielded a mutation rate, μ, of $0{\cdot}17 \times 10^{-9}$; those at 25° a rate of $0{\cdot}71 \times 10^{-9}$; and those at 37° a rate of $1{\cdot}6 \times 10^{-9}$.

Table 3 and Figs. 1 and 2 show the relation to temperature of the rate of death and of the rate of mutation/hr. in the stationary phase. At 0° no growth occurred so that death at that temperature was determined with cultures grown at 37° and then transferred to 0°, while mutation was investigated by transferring back again to 37°. Although more than 40% of the organisms were still surviving at the time of the last transfer, no mutations could be detected in addition to those which had occurred during the initial growth at 37° and those which occurred continuously upon return to 37° at a rate characteristic of that temperature ($1{\cdot}0 \times 10^{-9}$). The data in Table 3 are the average of three experiments at each temperature; the time periods studied averaged 239 hr. and covered 167–434 hr.

Table 3. *Summary of the average rates of death and of mutation in the stationary phase at different temperatures*

Temp.	Constant for the rate of death ($\times 10^{-3}$)	Range ($\times 10^{-3}$)	Rate of mutation/ bacterium/ hr. ($\times 10^{-9}$)	Range ($\times 10^{-9}$)
0°	4·6	2·5–6·7	$<$0·0006	—
15°	6·3	4·2–10·0	0·12	0·07–0·17
20°	6·8	4·8–9·4	0·24	0·14–0·30
25°	7·8	5·0–12·0	1·2	0·71–1·4
30°	9·5	6·1–13·4	1·1	0·69–1·3
37°	14·2	7·7–18·0	1·4	0·96–1·9

Figure 3 shows graphically the relation to temperature of the rates of mutation during growth and during the stationary phase and, as well, of the rate of growth and the rate of temperature-sensitive death during the stationary phase. The latter statistic was calculated by subtracting the k value at 0° from the k value determined according to equation (1) for any temperature. Because of the scatter of the data it is not certain whether the relation is curvilinear, such as is frequently found for biological events, or whether there is a constant temperature coefficient such as a Q_{10} lying between 2 and 3. In any event it is not possible to reject the hypothesis that all four events have the same temperature relation.

The number of nuclei (Feulgen-positive bodies)/organism was determined during growth and in the early and late stationary phase at different temperatures. The organisms were fixed in osmic acid vapour, hydrolysed in HCl and stained with Giemsa by the usual techniques. The nuclei were usually easy to count in all organisms in contrast to the preparations reported previously (Ryan & Wainwright, 1954). The average number of nuclei was 1·8 with an insignificant variation from 1·5 to 2·1, irrespective of stage or temperature.

DISCUSSION

The results of these investigations on the effect of temperature on the natural rate of mutation/bacterium/hr. are in accord with the findings of Witkin (1953). This rate and the rate of growth have the same temperature relation; therefore, the rate of mutation/generation is the same at all temperatures. For this reason it might be suspected that mutations are caused by something that happens a given number of times/generation; e.g. mutations might be the result of errors in the replication of the genetic material. On the other hand, these findings are not necessarily, nor are those of Witkin, in disagreement with the report of Novick & Szilard (1950) that bacterial mutations occur at

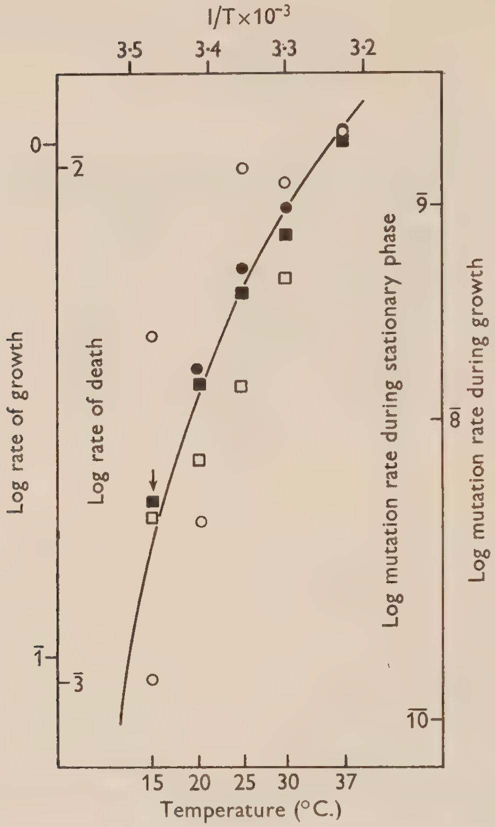

Fig. 3. The relation to temperature of the logarithms of the rates of mutation during growth (closed circles), of mutation during the stationary phase (open circles), of growth itself (closed squares), and of death during the stationary phase (open squares). The mutation rates are in terms of the chance of mutation/bacterium/hr; the growth rates are expressed as ln 2 divided by the generation time; and the death rates are the *k* values, calculated according to equation (1), from which the *k* at 0° was subtracted in order to compare just the temperature sensitive death with the rate of mutation. The curve was drawn by eye. If the value for mutation rate during growth at 15° is divided in half to correct for the presence of twice as many mutable units as usual, it falls to the point indicated by the arrow. The abscissa above measures the reciprocal of absolute temperature while that below is not exactly linear, because the reciprocal of absolute temperature is only approximately an inverse linear function of temperature in degrees Centigrade over this range (Belehradek, 1935).

rates which are independent of the length of generation time, the exceptions being very fast rates such as those which occur during unrestricted growth at 37° (Fox, 1955; Moser, 1958) and very slow rates where the generation time may be longer than 15 hr. and the organisms are frequently in the lag phase (Lee, 1953). It is conceivable that at each temperature the h^+ mutations occurred as a function of time, not growth rate; different rates of growth at a single temperature were not studied in the present case nor by Witkin, as was done in the experiments of Novick & Szilard. It would then be necessary to assume that the temperature coefficients of mutation and growth are identical either by coincidence or even because mutation is some function of division. As Novick (1956) pointed out, if mutations occurred at a constant rate/time unit during some critical period of division and if the length of this period were a constant proportion of the length of a generation, they would still be time-dependent. This might be the case if, for example, natural mutations were the result of DNA replication while adenine was in the rare tautomeric state or if they were a function of some metabolism that depended upon temperature but was independent of the limitations in nutrition used to change the rate of growth within the chemostat. But it is still a question whether in the present case and in that of Witkin there is a fixed chance of mutation/division at all temperatures or whether at a given temperature this chance is a function of the length of the generation time.

This problem has not been solved and the issue is further confused by technical difficulties. For example, it is necessary to compare mutation rates on a per mutable unit basis. If bacteria in the chemostats operating at different flow rates contain different numbers of mutable units, as nuclei or nuclear subunits, then the comparisons on a per bacterium basis would be confounded. By special methods the number of mutable units/h⁻ bacterium has been measured, but no direct attempts have been made to do the same with bacteria in the chemostat growing at different rates and mutating to phage resistance. Although indirect estimates may be made, they are uncertain and in conflict (cf. Fox, 1955; Novick, 1956, Fig. 5).

If mutations only occur during replication and mutation is a copy-error (Fabergé & Beale, 1941; Newcombe, 1953), then the fact that mutations occur in non-dividing bacteria requires an explanation. The metabolic activity of h⁻ bacteria in a histidine-limited stationary phase where there is no division is indicated by a rapid consumption of glucose (Ryan & Schneider, 1949). Thus there seems to be the possibility of a continued synthesis of genetic material, perhaps in the course of a metabolic turnover. This could conceivably be an unbalanced synthesis that leads eventually to death. Such a supposition is consistent with the probability that the rate of death and the rate of mutation in the stationary phase have the same Q_{10} as is indicated in Fig. 3. But unfortunately the lack of precision, and consequent difficulty in estimating the significance of small differences, does not allow the rejection of the interpretation that between 25° and 37° mutation in the stationary phase is essentially temperature independent.

It seems that little can presently be learned, as was once hoped (Timofeeff-Ressovsky, Zimmer & Delbrück, 1935) from an analysis of the temperature relation of mutation via the Arrhenius equation

$$k = Ae^{-W/RT}, \tag{6}$$

where k is some measure of rate, A a frequency factor, W the activation energy, R the gas constant and T absolute temperature. The activation energy, W, if constant over the temperatures studied, may be of the order of 0·5 eV. Since it is not even known as yet whether natural mutations are monomolecular or bimolecular or both, the significance of the frequency factor A is doubtful. As was the case with the mutation to phage T_5 resistance studied by Novick & Szilard (1950), a reversion from one homocysteineless condition in *Ustilago maydis* (Ishikawa & Tanaka, 1957) and mutation to the homocysteineless condition in an unstable strain of the same organism (Ishikawa, 1957), the A for mutation during growth is extraordinarily small. As a matter of fact, not only W but also A (5×10^{-4}/sec.) is almost the same for mutation to h^+ during growth as for mutation to T_5 resistance ($A = 10^{-3}$). In view of numerous uncertainties (Glasstone, Laidler & Eyring, 1941; Hinshelwood, 1951; see also the negative temperature characteristics of Fabergé & Beale, 1941 and Rhoades, 1941), the fact that the frequency factor A is small, or that it varies with conditions and is 2×10^{-2}/sec. for mutation to h^+ during the stationary phase, cannot assure us that mutation is or is not a monomolecular process nor that the activated state is of long duration.

The exact study of mutation demands a greater precision in measurement than is now available. Although Lederberg (1955) suggested that spontaneous mutations can be measured with a precision of 5–10 % with continuous flow devices such as the chemostat, a survey of the published data reveals discrepancies exceeding 30 % in mutation rates which should be identical. Rates determined by other methods may have a higher precision (Ryan, Schwartz & Fried, 1955). Further, the thermodynamic analysis of mutation requires more extensive experimentation (McElroy & Swanson, 1951) and needs be supplemented by other approaches.

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REFERENCES

Belehradek, J. (1935). *Temperature and Living Matter.* Berlin: Gebrüder Borntraeger.

de Rothschild, B. (1954). Mutant equilibrium in the chemostat. *Proc. 9th Int. Congr. Genetics*, pt. II, 977.

de Rothschild, B. & Ryan, F. J. (1953). Selective differences between new and old bacterial mutants. *Proc. 6th Int. Congr. Microbiol.* **1**, 726.

Fabergé, A. C. & Beale, G. H. (1941). An unstable gene in Portulaca: mutation rate at different temperatures. *J. Genet.* **43**, 173.

Fox, M. S. (1955). Mutation rates of bacteria in steady state populations. *J. gen. Physiol.* **39**, 267.

Glasstone, S., Laidler, K. J. & Eyring, H. (1941). *The Theory of Rate Processes.* New York: McGraw-Hill.

Hinshelwood, C. N. (1951). *The Structure of Physical Chemistry.* Oxford: Clarendon Press.

Ishikawa, T. (1957). An unstable strain of *Ustilago maydis. Jap. J. Genet.* **32**, 213.

Ishikawa, T. & Tanaka, N. (1957). Effects of temperature on reversion rate of homocysteineless mutants of *Ustilago maydis. Bot. Mag. Tokyo*, **70**, 183.

Kaplan, R. W. (1947). Spontane Mutabilität bei *Bacterium prodigiosum. Z. Naturf.* **2*b***, 308.

LEDERBERG, J. (1955). Genetics and microbiology. In *Perspectives and Horizons in Microbiology*, pp. 24–39, by Waksman, S. A. New Brunswick: Rutgers University.

LEE, H. H. (1953). The mutation of *E. coli* to resistant bacteriophage T_6. *Arch. Biochem. Biophys.* **47**, 438.

MCELROY, W. D. & SWANSON, C. P. (1951). The theory of rate processes and gene mutation. *Quart. Rev. Biol.* **26**, 348.

MOSER, H. (1958). The dynamics of bacterial populations maintained in the chemostat. *Publ. Carneg. Instn*, no. 614, 1–136.

NEWCOMBE, H. B. (1953). The delayed appearance of radiation-induced genetic change in bacteria. *Genetics*, **38**, 134.

NOVICK, A. (1956). Mutagens and antimutagens. *Brookhaven Symp. Biol.* **8**, 201.

NOVICK, A. & SZILARD, L. (1950). Experiments with the chemostat on spontaneous mutations of bacteria. *Proc. nat. Acad. Sci., Wash.* **36**, 708.

NOVICK, A. & SZILARD, L. (1953). Experiments on spontaneous and chemically induced mutations of bacteria growing in the chemostat. *Cold Spr. Harb. Symp. quant. Biol.* **16**, 337.

RHOADES, M. M. (1941). Genetic control of mutability in maize. *Cold Spr. Harb. Symp. quant. Biol.* **9**, 138.

RYAN, F. J. (1955). Spontaneous mutation in non-dividing bacteria. *Genetics*, **40**, 726.

RYAN, F. J., FRIED, P. & SCHWARTZ, M. (1954). Nuclear segregation and the growth of clones of bacterial mutants induced by ultraviolet light. *J. gen. Microbiol.* **11**, 380.

RYAN, F. J. & SCHNEIDER, L. K. (1949). The consequences of mutation during the growth of biochemical mutants of *Escherichia coli*. IV. The mechanism of inhibition of histidine-independent by histidineless bacteria. *J. Bact.* **58**, 201.

RYAN, F. J., SCHWARTZ, M. & FRIED, P. (1955). The direct enumeration of spontaneous and induced mutations in bacteria. *J. Bact.* **69**, 552.

RYAN, F. J. & WAINWRIGHT, L. K. (1954). Nuclear segregation and the growth of clones of spontaneous mutants of bacteria. *J. gen. Microbiol.* **11**, 364.

STOCKER, B. A. D. (1949). Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhimurium*. *J. Hyg., Camb.* **47**, 398.

TIMOFEEF-RESSOVSKY, N. W., ZIMMER, K. G. & DELBRÜCK, M. (1935). Über die Natur der Genmutation und der Genstruktur. *Nachr. Ges. Wiss. Göttingen, Fachgruppe*, **6**, N.F. 1, 189.

WITKIN, E. M. (1953). Effects of temperature on spontaneous mutations in *Escherichia coli*. *Proc. nat. Acad. Sci., Wash.* **39**, 427.

WITKIN, E. M. (1956). Time, temperature and protein synthesis; a study of ultraviolet-induced mutations in bacteria. *Cold Spr. Harb. Symp. quant. Biol.* **21**, 123.

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SMITH, E. E. B., MILLS, G. T., BERNHEIMER, H. P. & AUSTRIAN, R. (1959). *J. gen. Microbiol.* **20**, 654–669

A Study of some Enzymes in Extracts of a Non-Capsulated Strain of Pneumococcus Concerned with Uridine Pyrophosphoglycosyl Metabolism

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SUMMARY: Disruption of cells in a Mickle disintegrator in the presence of *n*-octanol is described as a method for obtaining extracts from the non-capsulated strain R19 of *Streptococcus pneumoniae*, derived from a strain of capsular type II. Such extracts were fractionated with ammonium sulphate in the presence of Celite and the distribution in the various fractions so obtained of a number of enzymes concerned with uridine pyrophosphoglycosyl metabolism was studied. Celite in the presence of ammonium sulphate was effective in removing organic pyrophosphatases, phosphatases acting upon uridine pyrophosphoglycosyl compounds and a reduced triphosphopyridine nucleotide (TPNH) oxidase.

A reduced diphosphopyridine nucleotide (DPNH) oxidase was concentrated by fractionation with ammonium sulphate and the uridyltransferases acting upon uridine pyrophosphoglucose (UPPG) and uridine pyrophosphoacetylglucosamine (UPPAG) were obtained as partially separated entities. Uridine pyrophosphogalactose (UPPgalactose) and guanosine pyrophosphomannose (GPPM) were not pyrophosphorylysed by the unfractionated material or by any of the fractions obtained.

UPPgalactose-4-epimerase and phosphogalactosyl uridyl transferase were present in the extracts under study, these enzymes being involved in the conversion of galactose-1-phosphate to glucose-1-phosphate. Such extracts also converted mannose to glucose phosphate without the participation of UPPG.

Evidence is provided for the presence of a diphosphopyridine (DPN) linked UPPG dehydrogenase which is capable of forming uridine pyrophosphoglucuronic acid (UPPGA) from UPPG. The UPPGA so formed was separated by ion-exchange chromatography. The extracts used did not catalyse the epimerization of UPPGA to uridine pyrophosphogalacturonic acid (UPPgalacturonic acid) as do extracts of a strain of capsulated type I pneumococcus. An examination of the cellular content of uridine nucleotides in relation to the bacterial growth curve showed that UPPGA did not accumulate in significant amounts until the organisms of this strain had reached the stationary phase.

In a comparison of the uridine pyrophosphoglycosyl metabolism of certain strains of capsulated and non-capsulated pneumococci (Smith, Mills & Harper, 1957*a*), cell-free enzyme systems were obtained by grinding the organisms in the presence of ballotini and buffers of specific pH values. The extracts so obtained were shown to contain the enzymes uridyl transferase, glucose-6-

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phosphate dehydrogenase, hexokinase, nucleoside diphosphokinase, phosphoglucomutase and inorganic pyrophosphatase. In a subsequent publication (Smith, Mills, Bernheimer & Austrian, 1958*a*) it was reported that, although disruption of the organisms with ballotini in the Mickle disintegrator caused considerable inactivation of many of the above enzymes, the addition of small amounts of *n*-octanol during such treatment considerably decreased this loss of activity. Extracts prepared in this manner from the non-capsulated strain R19 of *Streptococcus pneumoniae*, derived from a capsulated type II organism, were shown to convert UPPG to UPPGA (Smith *et al.* 1958*a*).

It is known that galactose and mannose are fermented actively by various strains of pneumococcus (Jürgens, 1937; Langvad-Nielsen, 1944) and also that galactose may be utilized by type III pneumococci for the synthesis of capsular polysaccharide (Bernheimer, 1953). In view of these findings, it was decided to investigate the enzyme systems involved in the metabolism of these two hexoses, with particular reference to their conversion to glucose or to glucose phosphates. To achieve this end, a method of fractionation of pneumococcal extracts has been developed and the separation of a number of enzymes concerned with uridine pyrophosphoglycosyl metabolism has been achieved.

METHODS

The organism used was a non-capsulated strain of pneumococcus, R19 (kindly supplied by Dr R. D. Hotchkiss, The Rockefeller Institute for Medical Research, New York, U.S.A.) derived from the capsulated type II strain, D39S. Glucuronic acid, glucose and rhamnose are the constituents of type II capsular polysaccharide (Butler & Stacey, 1955).

Uridine triphosphate (UTP) was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Adenosine triphosphate (ATP), uridine pyrophosphoglucose (UPPG), uridine pyrophosphoglucuronic acid (UPPGA), triphosphopyridine nucleotide (TPN, 75% purity), reduced TPN (TPNH, 75% purity) and α-galactose-1-phosphate were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A.

Diphosphopyridine nucleotide (DPN, 95% purity) and reduced DPN (DPNH, 65% purity) were obtained from C. F. Boehringer and Soehne, Mannheim, West Germany.

^{32}P labelled glucose-1-phosphate was prepared from ^{14}C labelled starch (Radiochemical Centre, Amersham, England) by the method of McCready & Hassid (1955) with rabbit muscle phosphorylase prepared by the method of Green & Cori (1943).

^{14}C labelled UPPG was prepared by the method of Munch-Petersen, Kalckar, Cutolo & Smith (1953) employing ^{14}C labelled glucose-1-phosphate, uridyl transferase prepared by the method of Munch-Petersen (1955) and inorganic pyrophosphatase prepared by the method of Heppel & Hilmoe (1955). The UPPG was separated by paper chromatography in the ethanol/ammonium acetate solvent of Paladini & LeLoir (1952).

Mannose-1-phosphate was synthesized by the method of Colowick (1938).

Bacto Brain Heart Infusion was obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

UPPG and uridine pyrophosphoacetylglucosamine (UPPAG) were prepared from yeast by the method of Caputto, LeLoir, Cardini & Paladini (1950) and separated by ion exchange and paper chromatography.

Guanosine pyrophosphomannose (GPPM) was isolated from yeast by the method of Cabib & LeLoir (1954).

UPPG dehydrogenase was isolated from calf liver by the method of Strominger, Maxwell, Axelrod & Kalckar (1957).

Cultivation and harvesting of pneumococci was carried out in the following manner. The organism was grown in 500 ml. Difco brain heart infusion medium (3·7 %, w/v; pH 7·4) containing 0·1 % Neopeptone. After incubation overnight, glucose was added to a final concentration of 1 % (w/v) and the lactic acid formed on subsequent incubation was neutralized with 3 N-NaOH according to the method described by MacLeod & Krauss (1947); all incubations were carried out at 36·5°. The organisms were collected by centrifugation at 1500 *g* for 20 min. at 0°, at a point in the logarithmic phase of growth determined by titration with NaOH. Figure 1 shows a typical growth curve for a standard inoculum of strain R19, expressed in terms of the amount of NaOH required for neutralization of the lactic acid produced; the points at which the organisms were harvested are shown in the figure.

Paper chromatography of nucleotides was carried out with the neutral ethanol/ammonium acetate solvent of Paladini & LeLoir (1952); sugar chromatography was carried out in the same ethanol/ammonium acetate solvent and in the butanol/acetic acid/water solvent of Partridge (1948) on Whatman no. 1 paper. The aniline hydrogen phthalate reagent of Partridge (1949) was used for the development of the sugar spots. Purine base and pyrimidine nucleotide chromatography were carried out in the *iso*propanol/HCl solvent of Wyatt (1951).

The method of Consden & Stanier (1952) was employed for the *ionophoresis of sugars* as borate complexes on Whatman no. 3 MM paper strips; ionophoresis of sugars was carried out also in 0·05 M-citrate buffer (pH 3·8) on Whatman no. 3 MM paper strips.

Assays of inorganic pyrophosphatase were conducted by the method of Heppel & Hilmoe (1951).

Assays of organic pyrophosphatase were carried out by allowing a suitable sample of the enzyme to react with UPPG and by measuring the glucose-1-phosphate liberated as described previously (Smith & Mills, 1954). When UPPAG and UPPGA were used as substrates for pyrophosphatase activity, the liberation of uridine-5′-monophosphate (UMP) was determined chromatographically.

Phosphatases acting upon UPPG, UPPAG and UPPGA were detected by the liberation of uridine pyrophosphate (UPP) which was located chromatographically.

Nucleoside triphosphatase activity was measured by allowing a suitable sample of the pneumococcal extract to react either with ATP or with UTP followed by measurement of the residual triphosphate as described previously (Smith & Mills, 1954).

Fractionation of extracts of Streptococcus pneumoniae, *strain* R19. The packed cocci obtained by centrifugation of a 500 ml. culture were suspended in 5 ml. M/15 phosphate buffer (pH 6·6) and subjected to disruption in a Mickle disintegrator at 0° for 30 min. in the presence of two drops of *n*-octanol and an amount of dry ballotini No. 12 equivalent to the volume of packed cocci. Before use, the ballotini were refluxed for 1 hr. with 2N-HCl, washed with tap water until free from acid, rewashed with several changes of distilled water and dried at 100°. Solid $(NH_4)_2SO_4$ was added to the suspension of disrupted pneumococci to a final concentration of 3M, followed by equilibration at 0° for 30 min. The precipitate so formed was adsorbed with Standard Super-Cel (Celite 519A, Johns-Manville Corporation) by adding 200 mg. Celite/10 ml. solution and allowing the mixture to stand at 0° for 30 min. before filtration. Filtration was carried out with suction at room temperature and the filter cake eluted for 30 min. by transferring it to 10 ml. ice-cold 2·5M-$(NH_4)_2SO_4$. The blended paste was refiltered with suction and subsequent elutions carried out as before with 10 ml. volumes of 2·0M, 1·5M and 1·0M-$(NH_4)_2SO_4$; 10 ml. distilled water was used as the final eluting agent. The amount of $(NH_4)_2SO_4$ in each fraction of eluate was adjusted to a final concentration of 3M with the solid salt, and after equilibration overnight at 0°, the resulting precipitates were collected by centrifugation at 18,000 *g* for 30 min. at 0°. Each precipitate was dissolved in the minimum volume of distilled water, dialysed overnight at 0° and the enzyme solutions so obtained adjusted to a standard volume of 2·5 ml. with distilled water and stored at −12°.

Protein nitrogen was determined by the method described by Mills (1948), the final protein estimation being carried out by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Enzymes in the unfractionated extracts of Streptococcus pneumoniae, *strain* R19

The presence of an extremely active DPNH oxidase in *S. pneumoniae* was reported previously (Smith *et al.* 1958*a*). Figure 2 indicates the activity of this enzyme in extracts obtained by disruption of strain R19 in a Mickle disintegrator. In this instance, the soluble extract was undialysed and the spectrophotometric assay of the oxidation of DPNH was carried out with the supernatant fluid after centrifugation of the disrupted cocci at 10,000 *g* for 20 min. An analogous assay of the oxidation of TPNH (Fig. 2) indicated the presence of an efficient TPNH oxidase.

Inorganic pyrophosphatase, organic pyrophosphatases capable of splitting UPPG, UPPAG and UPPGA to UMP, phosphatases acting on the same uridine pyrophosphoglycosyl compounds to yield UPP and nucleoside triphosphatases utilizing both UTP and ATP as substrates were readily demonstrable in the unfractionated extracts of pneumococci.

The distribution of various enzymes in fractionated extracts of Streptococcus pneumoniae, *strain* R19

The various fractions eluted from Celite with $(NH_4)_2SO_4$ will be referred to in the following manner. Eluates between 3·0 M and 2·5 M-$(NH_4)_2SO_4$ as *Fraction A*; 2·5 M and 2·0 M-$(NH_4)_2SO_4$ as *Fraction B*; 2·0 M and 1·5 M-$(NH_4)_2SO_4$ as *Fraction C*; 1·5 M and 1·0 M-$(NH_4)_2SO_4$ as *Fraction D*; 1·0 M-$(NH_4)_2SO_4$ and H_2O as *Fraction E*.

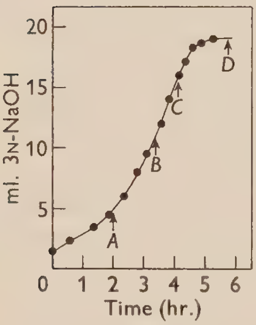

Fig. 1

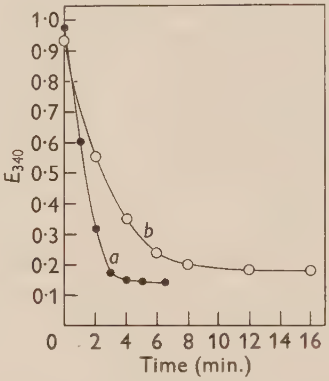

Fig. 2

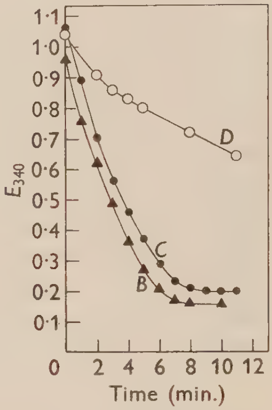

Fig. 3

Fig. 1. Titration with 3N-NaOH of lactic acid liberated by a culture of pneumococcus R19 following the addition of glucose. The total titration corresponds to an approximate tenfold increase in cell population. Cells were harvested at point C for the preparation of enzyme extracts and at points A, B, C and D for analyses of nucleotide content in relation to growth.

Fig. 2. The DPNH and TPNH oxidase activities of an unfractionated extract of cells of pneumococcus R19 obtained from a 500 ml. neutralized culture. The final volume of the extract was 4 ml. Curve (*a*): 25 µl. extract incubated with 0·13 µmole DPNH and 0·1 M-TRIS buffer, pH 7·8, to 1·0 ml. Curve (*b*): 30 µl. extract incubated with 0·12 µmole TPNH and 0·1 M-tris buffer, pH 7·8, to 1·0 ml.

Fig. 3. DPNH oxidase activity of fractions B, C and D obtained by Celite-$(NH_4)_2SO_4$ fractionation of disintegrated pneumococcal cells. 100 µl. extract incubated with 0·13 µmole DPNH and 0·1 M-tris buffer, pH 7·8 to 1·0 ml. Fractions A and E were inactive in this system.

DPNH and TPNH oxidases. It can be seen from Fig. 3 that the DPNH oxidase was located almost entirely in Fractions B and C. This distribution was consistent in several fractionations although slight activity was demonstrable occasionally in Fraction D. Fractions A and E were completely devoid of oxidase activity. While an active TPNH oxidase is present in the unfractionated extract of strain R19, none of the $(NH_4)_2SO_4$ fractions assayed acted upon TPNH either in the presence of or in the absence of DPNH. It would appear, therefore, that the oxidase in the fractions studied is specific for DPNH.

Inorganic pyrophosphatase, organic pyrophosphatases, phosphatases and nucleoside triphosphatases. Unfractionated extracts were rich in all these enzymes as was the total material precipitated with 3 M-$(NH_4)_2SO_4$. When Celite was used as the adsorbing agent under the conditions specified, however, inorganic pyrophosphatase, organic pyrophosphatases and phosphatases were retained by the Celite in the majority of fractionations and the $(NH_4)_2SO_4$ Fractions A, B, C, D and E were substantially free from these enzymes. Nucleoside triphosphatases utilizing both ATP and UTP as substrates appeared, however, in all fractions.

Uridyl transferases. Uridyl transferase activity was measured by the incorporation of ^{32}P labelled inorganic pyrophosphate (P–P) in accordance with the following reactions:

$$\text{UPPG} + {}^{32}\text{P}\text{–}{}^{32}\text{P} \rightleftharpoons \text{UP}^{32}\text{P}^{32}\text{P} + \text{glucose-1-phosphate}$$
$$\text{UPPAG} + {}^{32}\text{P}\text{–}{}^{32}\text{P} \rightleftharpoons \text{UP}^{32}\text{P}^{32}\text{P} + \text{acetylglucosamine-1-phosphate}$$

The method of assay was essentially that of Dr A. Munch-Petersen (personal communication, 1957) based upon that of Berg (1956). The nucleotides were adsorbed on pyrophosphate-washed Norite, washed with water and eluted with 50% (v/v) ethanol in water. For counting, a suitable sample of the Norite + aqueous ethanol suspension was plated. As further confirmatory evidence of uridyl transferase activity, the residual aqueous ethanol eluate was evaporated to a convenient volume and chromatographed in the neutral ethanol/ammonium acetate solvent of Paladini & Leloir (1952). In all instances in which the Norite + aqueous ethanol suspension showed isotopic activity greater than that of the control experiment, chromatography showed such activity to be localized entirely in UTP or UPP, the latter being derived from UTP by nucleoside triphosphatase activity.

In all experiments, chromatographically pure UPPG and UPPAG were used, care being taken to ensure that the former compound was free from UPPgalactose (Mills, Smith & Lochhead, 1957).

Typical values for the uridyl transferase activities of unfractionated extracts are shown in Table 1. It will be seen that the pyrophosphorolytic activity of such extracts is very similar when either UPPG or UPPAG is provided as the substrate. When an enzyme fraction eluted from the Celite filter cake between 3·0 M and 1·0 M-$(NH_4)_2SO_4$ was used, however, pyrophosphorolytic activity was more pronounced when UPPAG was used as the substrate. The results suggest that the uridyl transferases acting upon UPPG and UPPAG may be separable entities and attempts were made to fractionate the enzymes by means of the Celite/$(NH_4)_2SO_4$ technique described. Enzyme Fractions A, B, C and D were shown to have pyrophosphorolytic activity towards UPPG which activity could not be dissociated completely from the pyrophosphorolysis of UPPAG. Fraction E, however, showed specific pyrophosphorolytic activity towards UPPAG. The results are shown in Table 1.

Figure 4 shows the pyrophosphorolysis of UPPG by Fraction B and of UPPAG by Fraction D; the results are expressed as an ultraviolet absorption scan (260 mμ) of the chromatographed material. It will be seen that both Fractions B and D possessed considerable nucleoside triphosphatase activity towards UTP giving rise to uridine pyrophosphate (UPP) and UMP.

Table 1. *Pyrophosphorolysis of UPPG and UPPAG by whole and by fractionated extracts of* Streptococcus pneumoniae, *strain* R19

Chromatographically pure UPPG and UPPAG were used as substrates. 0·6 μmole of the requisite nucleotide was incubated with 1 μmole $MgCl_2$, 4 μmole inorganic pyrophosphate labelled with ^{32}P (2 μc), 100 μl. of the fraction indicated and 0·1 M-tris buffer, pH 7·8 to 1·0 ml., for 30 min. at room temperature. Controls run with no addition of nucleotide. The reaction mixtures were treated with perchloric acid (final concentration 1 %, w/v) and protein removed by centrifugation. Nucleotides were adsorbed on pyrophosphate washed charcoal (15 mg. per μmole nucleotide) for 10 min., the charcoal separated by centrifugation, washed twice with water and suspended in 1 ml. 50 % ethanol containing one drop of 5 % NH_4OH. 100 μl. of each suspension was plated, dried and the radioactivity determined with an end window counter. Results expressed as counts per minute (test minus control).

	Substrate	
Fraction	UPPG (c./min.)	UPPAG (c./min.)
Unfractionated R19 extract	18,000	16,000
3·0–1·0 M-$(NH_4)_2SO_4$ fraction (fractions A–D inclusive)	4,000	16,700
1·0 M-$(NH_4)_2SO_4$ to water (fraction E)	0	6,000

Metabolism of galactose and mannose

Because of the occurrence of galactose and mannose in the cellular polysaccharide of *Streptococcus pneumoniae* strain R19 (Smith, Mills, Harper & Galloway, 1957*b*), and because of the known ability of pneumococci to utilize both these sugars, experiments were carried out to determine the nature of the enzyme systems involved in the utilization of these two hexoses. Smith *et al.* (1957*a*, *b*), Glaser (1957), Glaser & Brown (1957), and Markovitz, Cifonelli & Dorfman (1958) have implicated uridine pyrophosphoglycosyl compounds in polysaccharide synthesis. It was considered desirable, therefore, to determine whether or not UPPgalactose and GPPM were pyrophosphorylysed by extracts of the organism under study.

UPPgalactose was isolated from a preparation of yeast containing both UPPG and UPPgalactose (Mills, Smith & Lochhead, 1957) by oxidizing the UPPG to UPPGA followed by separation of the residual UPPgalactose by chromatography. In experiments with such chromatographically pure UPPgalactose and with GPPM, no evidence was obtained for the formation of $UP^{32}P^{32}P$ or $GP^{32}P^{32}P$ respectively either by whole or by fractionated extracts of *Streptococcus pneumoniae*, strain R19.

The utilization of galactose by micro-organisms and mammalian tissues has been shown to follow the pathway of reactions outlined below (cf. Kalckar & Maxwell, 1958).

Galactose + ATP → galactose-1-phosphate + ADP (galactokinase)

Galactose-1-phosphate + UPPG ⇄ Glucose-1-phosphate + UPPgalactose (phosphogalactosyl uridyl transferase)

UPPgalactose ⇄ UPPG (UPPgalactose-4-epimerase)

The presence of the enzyme phosphogalactosyl uridyl transferase in the 3·0 M–1·0 M-$(NH_4)_2SO_4$ fraction is demonstrated clearly by the results presented in Fig. 5. From the data shown in Fig. 5, it is apparent that the presence of UPPG was necessary for the conversion of galactose-1-phosphate to glucose-1-phosphate; the latter was further converted to glucose-6-phosphate and subsequently oxidized to 6-phosphogluconic acid by the TPN-linked glucose-6-phosphate dehydrogenase of the pneumococci.

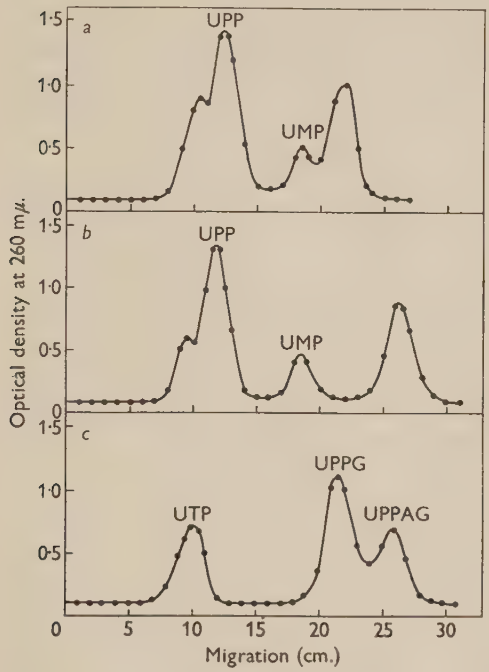

Fig. 4

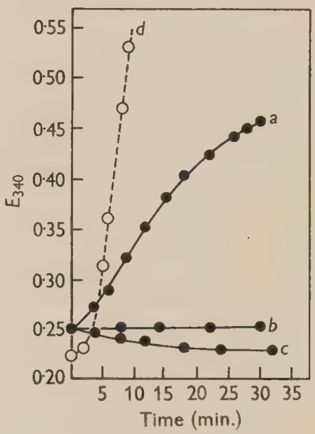

Fig. 5

Fig. 4. The pyrophosphorolysis of UPPG and UPPAG by ammonium sulphate fractions of an extract of *S. pneumoniae*, strain R19. Experimental details as in Table 1. The ethanol eluate from the charcoal was concentrated and chromatographed in the ethanol-ammonium acetate solvent, pH 7·5. The chromatograms were scanned in the Unicam S.P. 500 spectrophotometer at 260 mμ (cf. Smith & Mills, 1954). (*a*) UPPG and fraction B; (*b*) UPPAG and fraction D; (*c*) pure marker substances chromatographed simultaneously with (*a*) and (*b*).

Fig. 5. The phosphogalactosyl uridyl transferase activity of an ammonium sulphate fraction of an extract of *S. pneumoniae*, strain R19. (*a*) Complete system consisting of 0·1 μmole UPPG, 0·2 μmole α-galactose-1-phosphate, 0·25 μmole TPN, 5 μmole $MgCl_2$, 2·5 μmole cysteine, 100 μl. of a 3·0 M–1·0 M-$(NH_4)_2SO_4$ eluate fraction of R19 cells and 0·1 M-tris. buffer, pH 7·8, to 1·0 ml.; (*b*) complete system without UPPG; (*c*) complete system without α-galactose-1-phosphate; (*d*) same system but with 0·2 μmole α-glucose-1-phosphate in place of UPPG and α-galactose-1-phosphate.

The presence of UPPgalactose-4-epimerase in the extracts of strain R19 was demonstrated as follows. ^{14}C-labelled UPPG was incubated with the 3·0 M–1·0 M-$(NH_4)_2SO_4$ fraction in the presence of a small amount of DPN, followed by separation of the nucleotides by adsorption on charcoal, elution and chromatography in ethanol/ammonium acetate. The chromatographic area corresponding to UPPG was eluted, the sugars liberated by acid hydrolysis and identified by ionophoresis in borate buffer at pH 8·6. The positions of the sugar spots were located by the aniline hydrogen phthalate reagent and by autoradiography. In all such experiments glucose and galactose were identified and, in addition, a third radioactive reducing substance was invariably evident. This substance migrated in borate buffer to the same area as mannose. On chromatography in butanol/acetic acid/water, however, this substance did not occupy the position of mannose but had an $R_{glucose}$ value of 0·30 (mannose: $R_{glucose}$ = 1·25). At present the identity of this material is unknown.

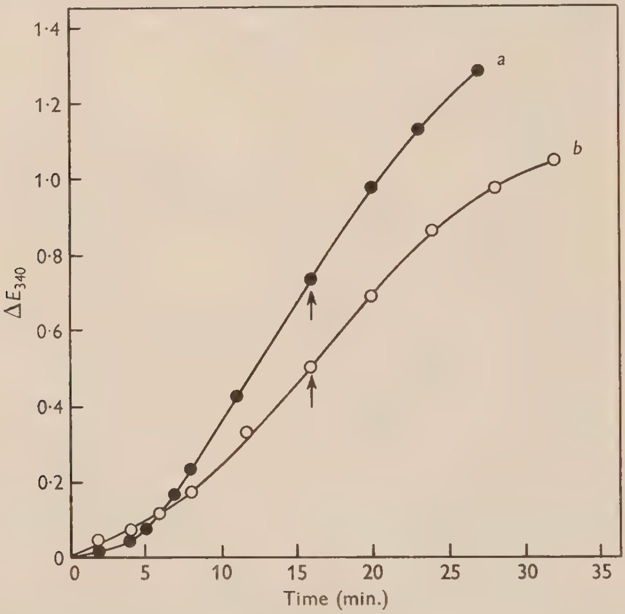

Fig. 6. The conversion of mannose and of mannose-1-phosphate to 6-phosphogluconic acid by an ammonium sulphate fraction of an extract of *S. pneumoniae*, strain R19. (*a*) 0·4 μmole mannose, 1·0 μmole ATP, 0·25 μmole TPN, 5 μmole $MgCl_2$, 2·5 μmole cysteine, 100 μl. of a 3·0 M–1·0 M-$(NH_4)_2SO_4$ fraction of an extract of R19 and 0·1 M-TRIS buffer, pH 7·8, to 1·0 ml. (*b*) As in (*a*) but with 0·2 μmole mannose-1-phosphate in place of mannose and ATP. ΔE_{340} values corrected for blanks run in the absence of mannose in (*a*) and mannose-1-phosphate in (*b*). 0·1 μmole UPPG was added at the points indicated by the arrows.

To date, it has not proved possible to demonstrate the presence of galactokinase in the extracts of strain R19 by means of spectrophotometric techniques.

Figure 6 shows the results of experiments on the conversion of mannose and of mannose-1-phosphate (M-1-P) to glucose phosphate with subsequent oxidation to 6-phosphogluconic acid. These reactions proceeded in the absence of added UPPG, and the addition of UPPG did not accelerate the reactions.

Berger, Slein, Colowick & Cori (1946) and Slein, Cori & Cori (1950) showed that mannose is phosphorylated by ATP in the presence of yeast and of mammalian hexokinase to give mannose-6-phosphate (M-6-P), and Slein (1950, 1954) described the conversion of M-6-P to glucose-6-phosphate, via fructose-6-phosphate. It would appear probable that this pathway is operative in *Streptococcus pneumoniae*, strain R19. LeLoir (1951) has shown that M-1-P is converted to M-6-P by phosphoglucomutase, a mechanism which may be operative in the present case.

The formation of UPPGA by fractionated extracts of Streptococcus pneumoniae *strain* R19

The inability to demonstrate spectrophotometrically the conversion of UPPG to UPPGA by extracts of *S. pneumoniae*, strain R19, results from the presence of DPNH oxidase (Smith *et al.* 1958*a*). In addition, it is not feasible to follow this reaction spectrophotometrically in fractionated extracts of strain R19 because the distribution of the DPNH oxidase during such fractionation follows the separation pattern of the enzyme which converts UPPG to UPPGA.

Smith *et al.* (1958*a*) demonstrated the formation of UPPGA from UPPG by a pneumococcal enzyme system which required the presence of DPN and, in this instance, the isolation and identification of the UPPGA was carried out by paper chromatographic techniques. The present experiment describes the isolation and identification of UPPGA by means of ion-exchange techniques.

Six μmole chromatographically pure UPPG were incubated with 5 ml. 0·1 M-tris (2-amino-2-hydroxymethyl-propane-1:3-diol) buffer (pH 9·0), 1 ml. 3·0 M–1·0 M-$(NH_4)_2SO_4$ fraction and 12 μmoles DPN for 30 min. at room temperature. At the end of this period the reaction was stopped by the addition of 10% (w/v) perchloric acid to a final concentration of 1% (w/v) and protein was removed by centrifugation at 4°. DPN and DPNH were removed from the deproteinized solution by passing it through a Dowex 50 H^+ column as described by Smith & Mills (1954) for the removal of AMP. The complete removal of DPN and DPNH was checked by spectrophotometric assay of the column effluent (Racker, 1950). The column effluent was adjusted to pH 9 with dilute NH_4OH and applied to a Dowex 1 Cl′ column (11 cm. × 1 cm.^{2}). Figure 7 indicates the results obtained by elution of the column with increasing concentrations of HCl and NaCl.

The materials eluted from the Dowex 1 Cl′ column were adsorbed on charcoal, eluted with ethanol and chromatographed in the ethanol/ammonium acetate solvent at pH 7. The material eluted in the 0·01 N-HCl/0·01 M-NaCl was mainly uridylic acid (UMP-5′). The component eluted in 0·01 N-HCl/0·03 M-NaCl chromatographed as UPPG. Hydrolysis of the chromatographic band corresponding to UPPG with 0·01 N-HCl for 15 min. showed the presence of UPP; the reducing components liberated by hydrolysis were three in number as determined by ionophoresis in borate buffer and chromatography in butanol/acetic acid/water. These components were identified as glucose, galactose and the unknown material referred to above. The component eluted from the column by 0·01 N-HCl/0·05 M-NaCl was identified as UPPGA (Smith & Mills, 1954; Smith *et al.* 1957*a*; Smith *et al.* 1958*a*). The only uronic acid present was glucuronic acid. This fact, and the observation that when pure UPPGA was incubated with a 3·0 M-1·0 M-$(NH_4)_2SO_4$ fraction there was no formation of UPPgalacturonic acid, indicates that *Streptococcus pneumoniae* strain R19 does not contain the UPPuronic acid-4-epimerase found in the capsulated type I strain studied by Smith, Mills, Bernheimer & Austrian (1958*b*).

It will be seen in Fig. 7 that the extent of conversion of UPPG to UPPGA was small; this observation, coupled with the marked instability of the UPPG dehydrogenase in the extracts used, suggested that there may have been considerable inactivation of the enzyme during preparation. It is well known that pneumococci actively produce hydrogen peroxide (McLeod & Gordon, 1922; cf. the presence of an active DPNH oxidase and the absence of the cytochrome system) and it was considered that this substance may be the inactivating agent. Preliminary experiments in which catalase was present throughout the procedures of fractionation and dialysis have shown increased yields of UPPGA. Further investigation of this problem is in progress.

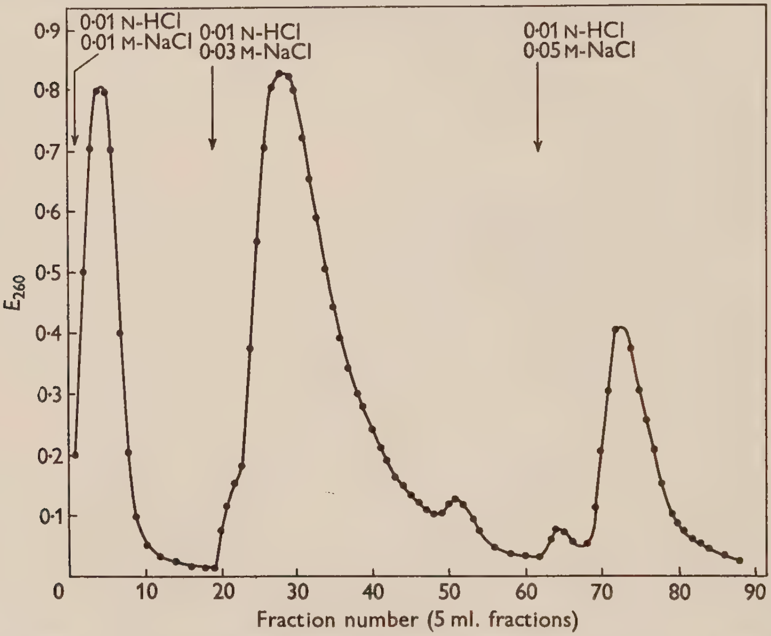

Fig. 7. Dowex 1 Cl′ column elution diagram showing the formation of UPPGA from UPPG by an ammonium sulphate fraction of an extract of *S. pneumoniae*, strain R19. See text for experimental details.

Stability of the enzyme fractions

The enzyme preparations obtained by $(NH_4)_2SO_4$ fractionation were most stable when stored at −12°. Under such circumstances, however, the DPNH oxidase lost 90 % of its original activity over a period of 2 weeks; uridyl transferase activity showed a decrease of 30 % after 1 week. The least stable of the uridine pyrophosphoglycosyl enzyme systems under these conditions was the UPPG dehydrogenase which showed complete loss of activity within 2 days.

Nucleotide analysis at various stages of growth

Four identical 500 ml. cultures of the strain R19 were incubated for 16 hr. at which time there were *c.* 10^9 cocci/ml. (by turbidimetry). Glucose was then added and the lactic acid formed during subsequent incubation was neutralized in the manner described. Growth was stopped at the four points indicated in Fig. 1, the number of cocci/ml. determined and a nucleotide analysis carried out on Dowex 1 Cl′ as described for Fig. 7, the nucleotides present in each fraction being identified by paper chromatography. The results are presented in Table 2.

Table 2. *Nucleotide analysis of* Streptococcus pneumoniae, *strain* R19, *at various stages of growth*

Total density units (D.U.) = $E_{260} \times$ volume.

Phase of growth (cf. Fig. 1)	Cells/ml.	0·01 N-HCl, 0·01 M-NaCl. Eluate	0·01 N-HCl, 0·03 M-NaCl Eluate		0·01 N-HCl, 0·05 M-NaCl Eluate	0·01 N-HCl, 0·20 M-NaCl Eluate
		Total D.U.	Total D.U.	Ratio: $\frac{\text{UPPG}}{\text{UPPAG}}$	Total D.U.	Total D.U.
A	$1{\cdot}5 \times 10^9$	76	20	0·20	17	15
B	$3{\cdot}1 \times 10^9$	122	59	0·42	19	8
C	$6{\cdot}3 \times 10^9$	176	83	1·84	6	7
D	$10{\cdot}0 \times 10^9$	400	188	1·30	43	88

A number of points emerge from these analyses. It will be noted that, in the 0·01 N-HCl/0·03 M-NaCl fraction, the UPPG/UPPAG ratio increased markedly up to point C and then fell slightly at point D. In the 0·01 N-HCl/0·05 M-NaCl and 0·01 N-HCl/0·2 M-NaCl fractions, there was no significant alteration in the nucleotide content until the resting phase D was reached; at this point the material accumulating in the 0·01 N-HCl/0·05 M-NaCl fraction was UPPGA and in the 0·01 N-HCl/0·20 M-NaCl fraction, the triphosphates UTP and ATP.

DISCUSSION

The preparation of extracts of pneumococci by their disruption in a Mickle disintegrator in the presence of *n*-octanol has made possible the identification and partial purification of a number of enzymes of *Streptococcus pneumoniae*, strain R19, which are concerned with uridine pyrophosphoglycosyl metabolism. From these and from other experiments, the pathways for the formation of UPPG and UPPgalactose have been established. The difficulties encountered in establishing the mechanism of formation of UPPGA in *S. pneumoniae*, strain R19, have resulted from the presence within this organism of a DPNH oxidase which does not permit the use of spectrophotometric assays. Also, very active organic pyrophosphatases and phosphatases in unfractionated extracts of strain R19 have caused a rapid breakdown of uridine pyrophosphoglycosyl substrates during attempts to follow the metabolism of these compounds. While direct fractionation of pneumococcal extracts with $(NH_4)_2SO_4$ does not alter the problem of the breakdown of such substrates, the use initially of Celite as an adsorbing agent under the conditions specified results in the almost complete elimination of interfering organic pyrophosphatases and phosphatases.

The conversion of UPPG to UPPGA by fractionated extracts of strain R19 is mediated through a DPN^+ linked system; in the absence of DPN^+ no formation of the uronic acid nucleotide from UPPG was observed. In view of the DPN^+-dependent pathway for the formation of UPPGA from UPPG established by Strominger, Kalckar, Axelrod & Maxwell (1954) and Strominger, Maxwell, Axelrod & Kalckar (1957) for liver tissue, it would appear that an analogous system is operative in the metabolism of the pneumococci used in the present work. The existence of an extremely active DPNH oxidase can be understood as having considerable importance in pneumococcal metabolism during UPPGA synthesis by providing a rapid cyclic method of replenishing DPN^+ from DPNH.

From the data presented, it appears that the uridyl transferases which utilize UPPG and UPPAG as substrates may be separable entities. The inability of strain R19 to pyrophosphorolyse GPPM suggests that mannose or a compound containing mannose may function as recipient during the synthesis of the cellular polysaccharide of the organism, the 'active transferable' sugar being derived either from UPPAG or from UPPgalactose.

It has been shown in the present work that mannose can be converted to glucose phosphate by extracts of *Streptococcus pneumoniae*, strain R19, without the participation of UPPG, whereas UPPG is essential for the conversion of galactose-1-phosphate to glucose-1-phosphate via the phosphogalactosyl uridyl transferase system. The direct formation of UPPgalactose from galactose-1-phosphate and UTP does not appear to be operative in this strain of pneumococcus.

The formation of glucuronic acid in *Streptococcus pneumoniae*, strain R19, appears to follow the pathway identified in animal tissues (cf. Mills, Lochhead & Smith, 1958) which includes the nucleotides UPPG and UPPGA. It is interesting to note that, although strain R19 does not produce capsular polysaccharide, there seems to be little or no impairment of the synthesis of glucuronic acid. It is likely, therefore, that the inability of strain R19 to form a capsule results from a mutation which affects the formation of rhamnose and/or one concerned with the polymerization of capsular polysaccharide. Rhamnose constitutes approximately 50% of type II capsular material; at present nothing is known concerning the synthesis of this methylpentose by pneumococci.

The results of the nucleotide analyses at various stages of growth show an increase in UPPG during the logarithmic phase followed by a decrease during the resting phase. The increased production of UPPG may be related to the necessity for the formation of UPPgalactose, the latter participating presumably in the synthesis of somatic C polysaccharide. A knowledge of the ratio of UPPG/UPPgalactose at this stage would elucidate this point more thoroughly.

The decrease in UPPG at the end of growth may be coupled with the increase of UPPglucuronic acid at this point. The appearance of UPPGA at this stage only, may indicate that, in the absence of the production of capsular polysaccharide, the synthesis of UPPGA is relegated to that of a minor pathway during the growth of this strain. The accumulation of triphosphates at the end of growth may be related to the decreased rate of formation of UPP-glycosyl compounds when growth ceases.

The preliminary experiments on the effect of catalase on UPPG dehydrogenase activity suggest inactivation of this enzyme by H_2O_2. The ability of sulphydryl compounds and of proteins to replace catalase is being investigated to ascertain the nature of this protective process.

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REFERENCES

Berg, P. (1956). Acyl adenylates: an enzymatic mechanism of acetate activation. *J. biol. Chem.* **222**, 991.

Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). Isolation of hexokinase from baker's yeast. *J. gen. Physiol.* **29**, 379.

Bernheimer, A. W. (1953). Synthesis of Type III pneumococcal polysaccharide by suspensions of resting cells. *J. exp. Med.* **97**, 591.

Butler, K. & Stacey, M. (1955). Immunopolysaccharides. Part IV. Structural studies on the Type II *Pneumococcus* specific polysaccharide. *J. chem. Soc.* p. 1537.

Cabib, E. & LeLoir, L. F. (1954). Guanosine diphosphate mannose. *J. biol. Chem.* **206**, 779.

Caputto, R., LeLoir, L. F., Cardini, C. E. & Paladini, A. C. (1950). Isolation of the coenzyme of the galactose phosphate-glucose phosphate transformation. *J. biol. Chem.* **184**, 333.

Colowick, S. P. (1938). Synthetic mannose-1-phosphoric acid and galactose-1-phosphoric acid. *J. biol. Chem.* **124**, 557.

Consden, R. & Stanier, W. M. (1952). Ionophoresis of sugars on paper and some applications to the analysis of protein polysaccharide complexes. *Nature, Lond.* **169**, 783.

Glaser, L. (1957). Enzymatic synthesis of cellulose by *Acetobacter xylinum*. *Biochim. Biophys. Acta*, **25**, 436.

Glaser, L. & Brown, D. H. (1957). The synthesis of chitin in cell-free extracts of *Neurospora crassa*. *J. biol. Chem.* **228**, 729.

Green, A. A. & Cori, G. T. (1943). Crystalline muscle phosphorylase. *J. biol. Chem.* **151**, 21.

Heppel, L. A. & Hilmoe, R. J. (1951). Purification of yeast inorganic pyrophosphatase. *J. biol. Chem.* **192**, 87.

Heppel, L. A. & Hilmoe, R. J. (1955). Inorganic pyrophosphatase. *Biochem. Prep.* **4**, 34.

Jürgens, A. (1937). Investigations regarding the fermentative ability of the Pneumococci. *Acta path. microbiol. scand.* **14**, 511.

Kalckar, H. M. & Maxwell, E. S. (1958). Biosynthesis and metabolic function of uridine diphosphoglucose in mammalian organisms and its relevance to certain inborn errors. *Physiol. Rev.* **38**, 77.

Langvad-Nielsen, A. (1944). Fermentation power of pneumococci. *Acta path. microbiol. scand.* **21**, 370.

LeLoir, L. F. (1951). The metabolism of hexosephosphates: in *A Symposium on Phosphorus Metabolism*, **1**, 67. Ed. McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

McCready, R. M. & Hassid, W. Z. (1955). α-D-Glucose-1-phosphate. *Biochem. Prep.* **4**, 63.

MacLeod, C. M. & Krauss, M. R. (1947). Stepwise intratype transformation of pneumococcus from R to S by way of a variant intermediate in capsular polysaccharide production. *J. exp. Med.* **86**, 439.

McLeod, J. W. & Gordon, J. (1922). Production of hydrogen peroxide by bacteria. *Biochem. J.* **16**, 499.

Markovitz, A., Cifonelli, J. A. & Dorfman, A. (1958). Biosynthesis of hyaluronic acid by cell-free extracts of group-A streptococci. *Biochim. biophys. Acta*, **28**, 453.

Mills, G. T. (1948). The β-glucuronidase activity of ox spleen and the assay of β-glucuronidase preparations. *Biochem. J.* **43**, 125.

Mills, G. T., Lochhead, A. C. & Smith, E. E. B. (1958). Uridine pyrophosphoglycosyl compounds and the formation of glucuronides by isolated enzyme systems. *Biochim. biophys. Acta*, **27**, 103.

Mills, G. T., Smith, E. E. B. & Lochhead, A. C. (1957). Presence of uridine pyrophosphogalactose and uridine pyrophosphogalactose-4-epimerase in non-galactose adapted yeasts. *Biochim. biophys. Acta*, **25**, 521.

Munch-Petersen, A. (1955). Investigations of the properties and mechanism of the uridine diphosphate glucose pyrophosphorylase reaction. *Acta chem. scand.* **9**, 1523.

Munch-Petersen, A., Kalckar, H. M., Cutolo, E. & Smith, E. E. B. (1953). Enzymic production of uridine triphosphate: Uridine diphosphoglucose pyrophosphorolysis. *Nature, Lond.* **172**, 1036.

Paladini, A. C. & LeLoir, L. F. (1952). Studies on uridine-diphosphate-glucose. *Biochem. J.* **51**, 426.

Partridge, S. M. (1948). Filter paper partition chromatography of sugars. *Biochem. J.* **42**, 238.

Partridge, S. M. (1949). Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* **164**, 443.

Racker, E. (1950). Crystalline alcohol dehydrogenase from bakers' yeast. *J. biol. Chem.* **184**, 313.

Slein, M. W. (1950). Phosphomannose isomerase. *J. biol. Chem.* **186**, 753.

Slein, M. W. (1954). Phosphohexose isomerases. *Fed. Proc.* **13**, 299.

Slein, M. W., Cori, G. T. & Cori, C. F. (1950). A comparative study of hexokinase from yeast and animal tissues. *J. biol. Chem.* **186**, 763.

Smith, E. E. B. & Mills, G. T. (1954). Uridine nucleotide compounds of liver. *Biochim. biophys. Acta*, **13**, 386.

Smith, E. E. B., Mills, G. T., Bernheimer, H. P. & Austrian, R. (1958*a*). The formation of uridine pyrophosphoglucuronic acid from uridine pyrophosphoglucose by extracts of a noncapsulated strain of pneumococcus. *Biochim. biophys. Acta*, **28**, 211.

Smith, E. E. B., Mills, G. T., Bernheimer, H. P. & Austrian, R. (1958*b*). The presence of an uronic acid epimerase in a strain of pneumococcus type I. *Biochim. biophys. Acta*, **29**, 640.

SMITH, E. E. B., MILLS, G. T. & HARPER, E. M. (1957*a*). A comparison of the uridine pyrophosphoglycosyl metabolism of capsulated and non-capsulated pneumococci. *J. gen. Microbiol.* **16**, 426.

SMITH, E. E. B., MILLS, G. T., HARPER, E. M. & GALLOWAY, B. (1957*b*). The cellular polysaccharide of a type II non-capsulated pneumococcus. *J. gen. Microbiol.* **17**, 437.

STROMINGER, J. L., KALCKAR, H. M., AXELROD, J. & MAXWELL, E. S. (1954). Enzymic oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid. *J. Amer. chem. Soc.* **76**, 6411.

STROMINGER, J. L., MAXWELL, E. S., AXELROD, J. & KALCKAR, H. M. (1957). Enzymatic formation of uridinediphosphoglucuronic acid. *J. biol. Chem.* **224**, 79.

WYATT, G. R. (1951). Recognition and estimation of 5-methylcytosine in nucleic acids. *Biochem. J.* **48**, 581.

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The Effect of Non-Lethal Deflagellation on Bacterial Motility and Observations on Flagellar Regeneration

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SUMMARY: The flagellation and motility of *Salmonella typhimurium* were unaffected by proteolytic enzymes; but when washed stationary-phase bacteria were briefly exposed to acid (e.g. pH 2·6 for 30 sec. at 43°) the proportion of motile, and of flagellated, bacteria was decreased from more than 50 % to less than 1 %, with little or no decrease in viable count. When *S. typhimurium* or *Vibrio metchnikovi* grown on stiff agar were rubbed for several minutes on the agar, the proportion of motile and of flagellated bacteria was decreased from *c.* 90 % (found in control bacteria, soaked off without rubbing) to *c.* 1 %, without decrease in viable count. High-speed stirring in a blendor also deflagellated and destroyed the motility of *S. typhimurium* in suspension, without killing the bacteria or retarding their growth. Blendor treatment sufficient to deflagellate *S. typhimurium* had no effect on the movements of certain non-flagellated motile organisms (*Leptospira canicola*, *Treponema pallidum* and *T. duttoni*), except when it caused gross morphological changes, attributed to cell damage. When log-phase broth cultures of *S. typhimurium* deflagellated in the blendor were incubated, bacteria with flagella (at first all short) and motile bacteria soon reappeared. At first many bacteria rotated; this suggests that flagella shorter than *c.* $0{\cdot}3\,\mu$ cause rotational, but not translational, motility. The rate of increase of mean summed flagellar length/bacterium was compatible with the hypothesis that flagella are not (spontaneously) shed in broth cultures of *S. typhimurium* and that the rate of growth of flagella is unaffected by deflagellation. Up to one generation time after deflagellation the mean number of flagella/bacterium was, however, less than before deflagellation. It is inferred that in a log-phase culture about half the flagella present at any one moment are 'fully-grown', incapable of being regenerated when broken off. All the results obtained are compatible with the hypothesis that the motility of flagellated bacteria is due to active movement of their flagella.

During work on the genetics of motility and flagellation in salmonella a non-lethal method of removing flagella from bacteria was needed. No satisfactory way of stripping selected individual bacteria of their flagella has been found, but it appeared that whole cultures might be deflagellated, without killing the bacteria, by several different methods. Such methods should provide a test of the hypothesis that the motility of flagellated bacteria results only from the active movement of their flagella, for total removal of the flagella would then necessarily result in loss of motility; whereas if normal motility in salmonella, etc., results from alterations of the shape of the bacterial body (Pijper, 1948, 1957), a treatment which removed all flagella might still leave motility unaffected. The effect of the application of similar treatment to motile bacteria which do not possess flagella (*Leptospira*, etc.) was also investigated, as a partial 'control' on the effects, other than deflagellation, of one of the treatments used. The manner and rate of the reappearance of flagellar in deflagellated salmonella cultures was investigated, both in respect of the correlation of flagellation with motility, and as a means of studying the method by which flagella are produced by bacteria.

METHODS

Organisms and media. In most experiments strain LT2 of *Salmonella typhimurium* was used; to obtain highly motile cultures, it was inoculated on to a semi-solid medium and recovered from the advancing edge of the spreading growth produced at 37°. Nutrient broth and agar were prepared from a tryptic digest of beef, or from a papain digest of horse meat. Except where otherwise stated, cultures were incubated statically at 37°.

Examination of flagella. Bacterial suspensions were fixed, washed, mounted, stained and counterstained by the method of Leifson (1951), with the modification of Quadling (1958). In some experiments, the fixed bacteria were washed with distilled water before staining, not by centrifugation as in Leifson's method, but by washing on the surface of a membrane filter. The stained films were examined microscopically; phase-contrast illumination facilitated the detection of non-flagellated bacteria. To determine the proportion of flagellated bacteria and the average no. flagella/bacterium, every bacterium encountered during several scans across a well-stained part of the film was examined and its flagella, if any, were counted. To avoid confusion with non-specific stain granules only filaments of a certain minimum length (generally one eye-piece micrometer unit, i.e. $0{\cdot}72\ \mu$) were reckoned as flagella. The lengths of individual flagella, i.e. distance in a straight line from tip to point of attachment, were measured with an eye-piece micrometer scale, every flagellum of each of a series of bacteria being measured for the calculation of the average flagellar length; these measurements were necessarily of low precision.

Determination of proportion of motile bacteria. In experiments on salmonella, this was done by low-power dark-ground microscopy of wet preparations, generally in a Helber counting chamber (hanging drops are not suitable for this method of illumination). Bacterial suspensions were usually examined immediately; when this could not be done, they were either stored at 4° or a bacteriostatic concentration of chloramphenicol was added, since this compound had been found to prevent synthesis of flagella without affecting bacterial motility. It has been found (Stocker, unpublished) that salmonella and other flagellated bacteria when well washed in saline, Ringer's solution or similar salt solutions, lose their motility (though not their flagella), but rapidly regain it when broth, any amino acid, bovine serum albumin, or a high concentration of phosphate are added. To avoid any risk of such 'paralysis', broth was always added to bacteria suspended in inorganic solutions before examination for motility. When a large proportion of the bacteria were motile, every bacterium crossing the central part of the field during a rapid scan was recorded as motile (either in translational movement or rotating) or non-motile; this scoring was difficult, and the results recorded should be regarded only as rough approximations. When only a very small proportion of the bacteria were motile the average number of bacteria in a block of counting chamber squares was first determined, and then several such blocks were rapidly searched for motile bacteria.

RESULTS

Deflagellation by chemical methods

Since it is known that the flagella of bacteria of several genera consist of protein (Weibull, 1949), pure trypsin, pure chymotrypsin and several crude preparations containing proteolytic enzymes were tested on live and killed *Salmonella typhimurium*. These agents had no action on the flagella as judged by flagellar staining; bacterial motility was also unaffected. Preliminary denaturation by heat or urea might have increased the susceptibility of the flagella to proteolytic enzymes, but as these treatments might be lethal they were not used.

Weibull & Tiselius (1945) and Weibull (1948) found that purified flagella from bacteria of several genera rapidly disintegrated at pH 3–4. Duncan (1935) reported that salmonella suspensions treated with dilute mineral acid were no longer agglutinable by anti-H sera, and that when live bacteria were treated with an amount of acid just sufficient to destroy their H-agglutinability their viability was little affected. These observations suggested that it might be possible to destroy the flagella of live bacteria by short exposure to acid without killing them. Preliminary experiments showed that at pH values 2·7–3·1 *Salmonella typhimurium* cultures in the logarithmic phase of growth were killed about four times as fast as stationary-phase cultures. Agar slope cultures incubated overnight at 37° were therefore used. The bacteria were washed off, and washed once in distilled water on the centrifuge to remove traces of broth which might have had an unwanted buffering action. Meynell (1955) showed that the bactericidal action of citrate + phosphate buffers was the same as that of hydrochloric acid solutions of the same pH value; citrate + phosphate buffers, of molarity about 1/7, were therefore used. Since the rate of lysis of flagella by acid is temperature-dependent (Weibull & Tiselius, 1945) the acid treatment was done at *c.* 44°. Early experiments suggested that exposure to pH values in the range 2·2–3·2 for about 1 min. might destroy the flagella without killing the bacteria. Samples of a dense suspension of washed *S. typhimurium* were therefore added to tubes of buffer solutions of nominal pH values 2·4, 2·6, 2·8 and 3·0. The treatment was terminated after $\frac{1}{2}$, 1 or 2 min. by adding a large volume of neutral phosphate buffer. Viable counts and determinations of the proportion of motile and flagellated bacteria were then made from certain of the treated suspensions, and from a control sample treated in neutral buffer instead of in acid. It will be seen (Table 1) that $\frac{1}{2}$ min. at pH 2·4 or 2·6, or 2 min at pH 2·8, decreased the proportion of bacteria with flagella detectable by staining from *c.* 64 % to less than 1 %. The viable count was only slightly decreased by these treatments. The proportion of bacteria which showed translational motility was correspondingly depressed to less than 1 %, as would be expected if only flagellated salmonella can swim.

In the pH 3·0 buffer deflagellation was less rapid than at lower pH values and the proportions of motile and flagellated bacteria were 30 and 32 %, respectively, after ½ min. exposure; and 3 and 9 % after 1 min. exposure.

Table 1. *Viability, flagellation and motility of stationary-phase* Salmonella typhimurium *after acid treatment*

The growth on overnight nutrient agar slopes of *Salmonella typhimurium* strain LT2 was washed off in distilled water; 0·1 ml. of dense suspension (*c.* 4×10^{10}/ml.) was added to 0·9 ml. of acid buffer at 43°. After the indicated time 9 ml. of pH 7·1 buffer was added. Flagellation determined on formalin-fixed samples, motility and viable counts on samples diluted in broth.

Treatment				Flagellation†			
pH value	Time (min.)	Viable count (proportion of control)	Motility* (proportion of bacteria motile)	Proportion of bacteria with flagella	Mean no. flagella/bacterium	Mean no. flagella/flagellate bacterium	Mean flagellar length (μ)
2·4	½	0·75	< 0·001	< 0·01	—	—	—
	1	0·23	—	—	—	—	—
	2	0·011	—	—	—	—	—
2·6	½	1·0	0·001	< 0·01	—	—	—
	1‡	0·72	< 0·001	—	—	—	—
	2	0·26	—	—	—	—	—
2·8	1	—	0·0005	—	—	—	—
	2	0·65	—	< 0·01	—	—	—
3·0	½	—	0·3	0·32	0·4	1·3	4·3
	1	—	0·03	0·09	0·13	1·5	3·4
	2	0·79	0·006	—	—	—	—
7·1	2‡	1·0	—	0·64	0·96	1·5	6·4

* Only bacteria showing translational movement were recorded as motile.

† Only filaments at least 0·7 μ long were recorded as flagella.

‡ From each of these tubes two motile bacteria were isolated by micro-manipulation; the resulting cultures were later tested for acid-resistance.

In films made from suspensions which had been incompletely deflagellated (e.g after 1 min. at pH 3·0) the average number of flagella/flagellated bacterium was about the same as in the control. This suggests that the original bacterial suspension was perhaps heterogeneous in respect of the acid-lability of the flagella of individual bacteria. Similarly, the very small number of motile bacteria found in some preparations were presumably bacteria with unusually acid-resistant flagella. To test whether such bacteria produced progeny with acid-resistant flagella, individual motile bacteria were isolated by micro-manipulation from some of the treated preparations, by the method of preliminary concentration by centrifugation and a 'trapping' drop (Stocker, 1956*b*) to facilitate the detection of a very small minority of motile organisms. Cultures grown from two motile organisms isolated from a suspension treated in pH 2·6 buffer for 1 min. were compared with two control cultures grown from single organisms isolated from the control suspension and not treated with acid. All four cultures were washed and given a standard acid treatment;

the extent to which motility was lost was about the same in all. Thus the minority of bacteria which remained motile after acid treatment were not mutants with acid-tolerant flagella.

Deflagellation by mechanical methods

Although it is known that flagella may be removed from some bacteria by vigorous shaking or repeated pipetting of dense suspensions neither method was found satisfactory for the present purpose. In particular even prolonged shaking of a *Salmonella typhimurium* suspension on a mechanical shaker at about 200 strokes/min. had but little effect on the proportion of motile bacteria. However, it was observed that only a small proportion of the bacteria were motile in suspensions of *S. typhimurium* harvested from the surface of nutrient agar plates by scraping off the growth with a glass rod, whereas when the bacteria were washed off the agar a high proportion were motile. This suggested that rubbing the film of bacterial growth over the surface of agar might detach the flagella. Experiments showed that the loss of motility was most complete when rather stiff nutrient agar (e.g. 3% (w/v) New Zealand agar) was used, and that after a period of rubbing sufficient to destroy motility very few or no flagellated bacteria were visible in stained films. To see whether the rubbing killed the bacteria, as well as deflagellating them and destroying their motility, bacteria were washed off two different portions of a single tray of stiff nutrient agar, either without rubbing or after several minutes rubbing to and fro over the agar surface with a bent glass rod. The suspensions of rubbed and control bacteria were diluted and their exact turbidities were measured; counts of viable bacteria were then made, and the proportions of motile and of flagellated bacteria were determined on live and stained preparations, respectively. From the results of the experiment (Table 2), it may be inferred that rubbing decreased the proportion both of flagellated and of motile bacteria from 90% or more to about 1%, without decreasing to a detectable extent the number of viable bacteria/'turbidity unit'. A similar experiment was done on *Vibrio metchnikovi*, grown on stiff

Table 2. *Effects of rubbing on stiff agar on the viability, motility and flagellation of a culture of* Salmonella typhimurium

S. typhimurium strain LT2 was grown for *c.* 18 hr. at 30° on stiff nutrient agar in a large tray. One part of the agar was cut out, and the bacteria washed off; the growth from another part was scraped together and rubbed over the agar for 3 min. and then washed off. The motility, flagellation, viable count and turbidities of the two suspensions were then measured.

Treatment	Viable count of suspension of turbidity 0·1 (arbitrary unit)	Proportion of bacteria motile		Flagellation	
		Translational	Rotating	Prop. of bacteria with flagella	Mean no. flagella/bacterium
Washed off	6·6 × 10^7/ml.	*c.* 0·9	—	0·98	7·4
Rubbed for 3 min. then washed off	6·9 × 10^7/ml.	0·006	0·004	0·016	0·092

nutrient agar at 30° for 18 hr. The suspension of the washed-off organisms contained *c.* 60 % of motile (translating) and *c.* 96 % of flagellate bacteria (about half of these having a flagellum at each pole); a suspension of the growth which had been rubbed over the agar for 3 min. contained less than 1 % of bacteria in translational movement and only 5 % of flagellated bacteria, those seen having each one very short flagellum only. The viable count was not significantly decreased.

The few flagellated bacteria which were detected in stained films of the rubbed bacteria (Table 2) included some with several long flagella; these resembled untreated bacteria. These unaffected bacteria were presumably those detected as normally motile in wet preparations. Their retention of flagella probably resulted from accidental escape from rubbing, perhaps in a mass of bacteria on the upper surface of the glass spreader, and not from any exceptional resistance to deflagellation, as in the case of the few motile bacteria in acid-treated suspensions.

Table 3. *Effect of blendor treatment of varying duration on the motility, flagellation and viable count of a suspension of* Salmonella typhimurium

A log-phase 37° broth culture of *S. typhimurium* strain LT2 was centrifuged, and the bacteria were suspended in quarter-strength Ringer's solution. 10 ml. portions were blended for the indicated times, and immediately examined for viable count and motility, and fixed for staining.

Duration of treatment (sec.)	Viable count/ml.	Proportion of bacteria motile		Proportion of bacteria flagellated*
		Translational	Rotating	
0	$8{\cdot}4 \times 10^7$	0·90	—	0·92
15	—	0·44	0·36	0·50
45	$11{\cdot}2 \times 10^7$	0·09	0·51	0·22
120	$9{\cdot}7 \times 10^7$	0·01	0·16	0·16
360	$10{\cdot}7 \times 10^7$	0	0·15	0·08

* Only bacteria with one or more flagella of length at least 3 micrometer units (2·1 μ) were scored as flagellated.

Violent stirring of a bacterial suspension by a high-speed propeller in a 'blendor' has been shown to remove the filamentous appendages known as 'fimbriae' from a fimbriate strain of *Escherichia coli* (Brinton, Buzzell & Lauffer, 1954) and to detach the head membranes of adsorbed phage particles (Hershey & Chase, 1952), in each case without destroying the organisms. Experiments showed that treatment of suspensions of *Salmonella typhimurium* for a few minutes in an M.S.E. blendor (Measuring and Scientific Equipment Ltd., Spenser Street, London, S.W. 1) with the 'universal container' type of bottle (capacity *c.* 25 ml.) resulted in almost complete deflagellation and loss of motility, without detectably lowering the viable count. Table 3 records the results of one experiment. It will be seen that treatment for 45 sec. sufficed to decrease the proportion of flagellated bacteria from about 92 to 22 %, and of bacteria which showed translational motility from about 90 to 9 %; treatment for 120 sec. further decreased these proportions to 16 and 1 %; but even treatment for 360 sec. caused no decrease in viable count.

Similar experiments showed that a period of blending which decreased the proportion of flagellated, and of motile, bacteria in a log-phase broth culture to zero did not retard subsequent growth, as judged by the comparison of viable counts and turbidity with those of untreated portions of the same suspensions.

The proportion of bacteria rendered non-motile by a standard treatment in the blendor varied from experiment to experiment. On some occasions only 1 % or less of the treated bacteria showed any movements detectably greater than Brownian, whereas in other experiments, e.g. that recorded in Table 3, many of the treated bacteria were seen to be spinning, but not progressing. Staining of samples from such preparations showed the presence of many bacteria with very short flagella, presumably stumps left by the breaking-off of the distal part of originally longer flagella. In an attempt to discover the means by which blending removed the flagella, the effect of various factors on the extent of loss of motility was investigated. Portions of a single suspension were blended for 10 sec. at concentrations of 5×10^{10}, 5×10^{9} and 5×10^{8} bacteria/ml.; in all three samples the proportion of motile bacteria was decreased about equally, i.e. from *c.* 80 to 5–10 %. It may be inferred that the loss of flagella did not result from the collision of one bacterium with another, or with the flagella of another. Loss of motility was more nearly complete when the volume blended in a 'universal container' was 10 ml. (depth of liquid *c.* 25 mm.) rather than 20 ml., presumably because the shearing motion in the upper part of the suspension, i.e. further from the stirring blades, was less intense. It was also more nearly complete when the bacteria were in suspension in normal saline or in Ringer's solution instead of in broth, probably because in the latter medium bacteria escaped from the shearing field by entrapment in the froth which formed as soon as blending was begun. Blending was more effective at lower temperatures (4° better than room-temperature, and room-temperature better than 37°), presumably because of the greater viscosity of water at lower temperatures. The actual speed of the propeller shaft during blending treatment was found to be about 12,000 rev./min. and to be about the same for broth or water. A short treatment, insufficient for complete deflagellation, much decreased the average length of the flagella. This suggests that blending removed flagella by repeated breaking-off of the distal parts of the flagella rather than by breaking them off at the base. The persistence of a minority of rotating bacteria, and of bacteria with very short flagella, even after periods of blending several-fold longer than that which sufficed to remove the greater part of the flagella (Table 3) suggests that the probability of a flagellum being broken off during blending is inversely related to its length.

The blending method has been applied to flagellated bacteria of several genera. In an experiment with a strain of *Brucella bronchiseptica,* blending in broth for 60 sec. decreased the proportion of bacteria in translational movement from *c.* 40 to *c.* 0·2 % and greatly decreased the proportion of flagellated bacteria without affecting the viable count. It was also observed that blending destroyed the motility of suspensions of a Sarcina, a Pseudomonas and a strain of *Spirillum mancuniense,* the latter being a free-living spirillum with tufts of flagella at one or both poles (Cayton & Preston, 1955). Viable counting and flagellar staining was not done in these cases.

To test the effect of blending on the H-agglutinability of the bacteria a broth culture of *Salmonella typhimurium* in phase 2 was divided into two parts, one of which was blended. Both parts were formalinized and tested for agglutinability by an anti-H serum (anti-1, 2, 3). The unblended portion was agglutinated in typical floccules, while the blended sample showed very fine shreds only. One washing of the formalized suspensions did not diminish the agglutinability of the unblended sample; the washed blended suspension showed no detectable agglutination. Presumably, therefore, the agglutinates in the blended suspension consisted of detached flagella, perhaps with some entangled bacterial bodies. These results would be expected if the H-antigen of Salmonella were present only in the flagella, and if the latter were detached by blending. These conclusions are similar to those of Craigie (1931) and correspond to the generally accepted view.

Reappearance of flagellate bacteria in deflagellated suspensions

When a stationary-phase broth culture of *Salmonella typhimurium*, deflagellated by blending, was re-incubated without change of medium, only a small proportion of motile bacteria appeared, and these only after a considerable delay. By contrast, when a broth culture in the exponential phase of growth was so treated, the proportion of motile bacteria (at first zero or nearly so) rapidly increased, and after one generation-time was about the same as in non-blended samples of the same culture. The motile organisms which first appeared were nearly all rotating, with little or no translational movement; as incubation continued the proportion of rotating bacteria increased, and then decreased as the proportion in translational movement increased. Staining of samples fixed at times when only rotational motility was obtained showed, as expected, only bacteria with short (or no) flagella. These results, together with those described above on the rotating movement of bacteria in incompletely deflagellated suspensions, suggest that a flagellum shorter than some critical length can rotate, but not propel, the bacterium to which it is attached. The data suggest that this critical length is about $2{\cdot}9\ \mu$. For instance, in one experiment at 36° at 18 min. after the end of blending the majority of the bacteria were rotating and a few were translating; in a stained film 14 of 20 bacteria scored had at least one flagellum four micrometer units ($= 2{\cdot}9\ \mu$) long, but only 6 of these 14 had any flagella five units or more in length.

The increase in average flagellar length on continued incubation of deflagellated bacteria suggested that the Salmonella flagella increased gradually in length, presumably as a result of the gradual secretion and extrusion of flagellum by the bacterium, perhaps through the activity of some sort of basal granule. It is clear that they are not extruded instantaneously, already formed and of fixed length. Different types of experiment (Stocker, 1956*a*, *b*; Quadling & Stocker, 1956 and unpublished; Quadling, 1958) indicated that in broth cultures the flagella of Salmonella were not shed (or were shed only very infrequently) but were retained and continued to function for several or many generations. This conclusion and the assumption that deflagellation of a bacterium does not alter the rate at which it produces flagella permit a calculation of the theoretical rate at which the average 'amount of flagellum' (i.e. summed lengths of all flagella)/bacterium will increase from zero towards its original value in a log-phase broth culture incubated after complete deflagellation. The value before deflagellation, i.e. the 'steady-state' value during exponential growth, may be taken as 1, in arbitrary units. At a time t generation-times after deflagellation the proportion of the total bacterial mass which has been produced since deflagellation will be $(2^t-1)/2^t = 1-t^{-2}$. The only flagella at this time will be those formed *pari-passu* with the increase in bacterial mass since deflagellation, and the average flagellar amount/bacterium will therefore also be $(1-t^{-2})$ of its value before deflagellation (1, in arbitrary units). This expression (plotted in Fig. 1) has value 0 when $t = 0$, and tends to 1 as t increases, i.e. as the effect of deflagellation is extinguished by subsequent growth. To test this prediction broth cultures of *Salmonella typhimurium* in the logarithmic phase of growth, at 25° or 37°, were used. A portion of the culture was treated in the blendor, and the treated and control portions of the culture were kept in logarithmic growth by dilution at intervals. Growth was followed by measurement of turbidity, or by viable counts. Samples were taken at intervals for flagellar staining, and the average no. flagella/bacterium and average length/flagellum were measured; their product gave the value of average flagellar amount/bacterium. Three experiments, the results of one of which are recorded in Fig. 1 and Table 4, gave similar results in that the values of average flagellar amount/bacterium at various times after deflagellation were in reasonable agreement with the predicted curve. Thus, these results, though only of low precision, lend some support to the assumptions made above, viz. that flagella are not shed and that their rate of formation is unaffected by deflagellation.

Table 4. *Reappearance of flagellation after blending in a log-phase* (25°) *broth culture of* Salmonella typhimurium

Part of a broth culture in log. phase of *Salmonella typhimurium* strain LT2 at 25° was blended for 5 min., the proportion of motile bacteria being thereby decreased from *c.* 0·8 to less than 0·01. At intervals samples were taken from the blended and unblended portions for staining and measurement of turbidity.

Sample	Average no. flagella / bacterium	Average flagellar length (μ)	Average flagellar amount/ bacterium (μ)
Unblended, 0 min.	4·5	3·7	16·8
0 min. after blending	0	—	0
20 min. after blending	2·6	1·7	4·5
40 min. after blending	3·8	1·9	7·1
60 min. after blending	3·7	2·4	8·9
80 min. after blending	4·5	2·5	11·2
100 min. after blending	4·2	2·7	11·4
Unblended, 100 min.	5·4	3·7	19·8

If in a log-phase culture every flagellum increased in length at a constant (linear) rate as a result of continuous secretion, all flagella broken off by blendor treatment should re-appear on subsequent incubation; and the average no. flagella/bacterium should remain constant, the average length increasing from zero towards its initial value as incubation continued. However, in all experiments the average no. flagella/bacterium in samples taken less than one generation-time after deflagellation was less than in the untreated controls. This deficit in average no. flagella/bacterium during the early stages of regeneration can be explained by the assumption that a flagellum, though it persists (and functions) for many generations, increases in length for only a limited period after its inception. If this be the case, some of the flagella in a culture at any moment will be growing (and will re-appear as short flagella soon after blending) and others will be fully grown flagella formed in earlier generations which once broken-off will not re-grow. The data suggest that rather more than half the flagella present in the cultures tested were not regenerated after blending. As most bacteria showed at least some flagella soon after blending, such non-regenerable flagella were not all carried by a fraction of

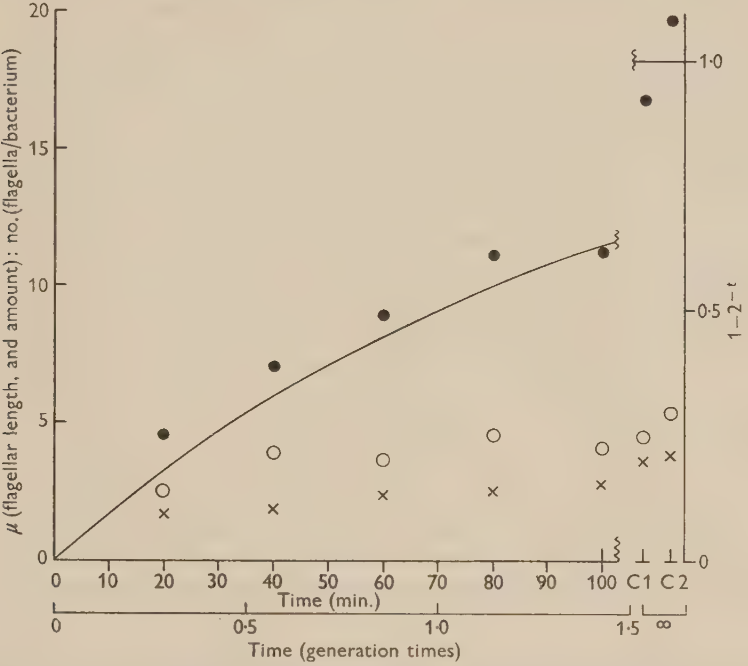

Fig. 1. Reappearance of flagellation after blendor treatment in a log-phase (25°) broth culture of *Salmonella typhimurium*. ○ = mean no. flagella/bacterium; × = mean length (μ)/flagellum; ● = mean flagellar amount/bacterium. C1 = observations on untreated portion of culture at time of treatment. C2 = observations on untreated portion at 100 min. Curve represents theoretical return of flagellar amount towards 18·3 μ (mean of C1 and C2 values) predicted by values of $1-2^{-t}$, where t is time (in generation-times) since treatment. Generation time, estimated from turbidity measurements, was 70 min.

the population with 'old' cell-walls. If this argument be correct, the average flagellar length in samples taken soon after deflagellation will be somewhat less than the product of the time since blending and the average rate of elongation of a flagellum during its phase of growth. In two experiments at *c.* 37° the average flagellar lengths at 9 min. (*c.* $\frac{1}{3}$ of a generation-time) were 1·1 μ and 1·3 μ, and in one experiment at 25° the average length at 20 min. (also *c.* $\frac{1}{3}$ of a generation-time) was 1·7 μ. These figures give rates of flagellar elongation of 0·12 and 0·14 μ/min. at 37°, and 0·085 μ/min. at 25°.

The correlation between flagellation and motility found in certain bacterial genera might indicate that flagella are a result of bacterial motility, rather than its cause (Pijper, 1946, 1957). We therefore compared the behaviour after blendor treatment of a non-motile flagellated ('paralysed') strain of *Salmonella typhimurium*, strain SW578 (Stocker, Zinder & Lederberg, 1953; Beighton, Porter & Stocker, 1958) with that of a motile mutant strain (SL272) obtained from it by selection in semi-solid medium. Both strains grew at the same rate (generation-time about 26 min. in broth at 35°), and both showed about the same degree of flagellar development. No bacterial movement other than Brownian was detected in the 'paralysed' strain, either before or at any stage after blending. Blending removed the flagella almost completely in both strains, and no difference was detected in the rate or manner in which flagellated bacteria re-appeared in the two strains.

Effect of blendor treatment on motile bacteria of non-flagellated genera

According to electron-micrograph evidence (e.g. Bradfield & Cater, 1952; Swain, 1955) Leptospira and spirochaetes of the relapsing fever and *Treponema pallidum* groups lack flagella of the ordinary kind, though the fibrils which are seen when the organisms are broken up bear some resemblance to flagella and perhaps constitute intracellular locomotor apparatus. It was therefore of interest to see whether treatment which sufficed to deflagellate salmonella and other flagellated bacteria would affect the characteristic movements of such non-flagellated organisms. Only blendor treatment was investigated. Because of shortage of material the 5 ml. blendor container was used in these experiments; special suspending media were needed to maintain the motility of the spirochaetes. Because of these differences of technique, and because it was found that prolonged treatment caused gross damage to some spirochaetes, a portion of a log-phase broth culture of motile *Salmonella typhimurium* was added to each suspension before treatment in the blendor, as a control on the efficacy of the treatment. Blood from a mouse inoculated 3 days earlier with a strain of *Treponema duttoni* was diluted in normal rat serum (to preserve the motility of the spirochaetes) and centrifuged for 5 min.; the supernatant fluid, containing a high concentration of motile spirochaetes, with motile *S. typhimurium* added, was blended for 2 min. The proportion of motile salmonella was decreased from 91 to 9%, and of motile treponemata from 96 to 43%. However, when thin films of the control and blended suspensions were examined by high-power phase-contrast microscopy, it was seen that many of the treponemata had been injured by the blending. In the controls more than

90% of treponemata appeared as regular helical filaments, whereas in the treated sample about half, including nearly all the non-motile ones, were abnormal in shape, appearing either as helical filaments with one or several rounded protrusions, or as irregular filaments, some showing protrusions. Thus, the motility of a spirochaete of the relapsing-fever type was less readily destroyed by blending than that of salmonella; and such loss of motility as occurred probably resulted from damage to the cell-wall which caused death.

A preliminary experiment with a culture of *Leptospira canicola* showed that blending for 1 min. had no effect on the motility of a suspension of these organisms in Ringer's solution; and that blending for 6 min. had a slight effect, the proportion of non-motile leptospira (neither 'rotating' about their long axis, nor translating) being increased from less than 5 to *c.* 30%. Many of the non-motile forms seen appeared to be broken. When a mixture of Leptospira and salmonella was blended, treatment for 2 min. decreased the proportion of motile salmonella from 80 to *c.* 4%, without noticeable effect on the motility of Leptospira. A suspension of *Treponema pallidum* from a rabbit lesion, prepared for use in the treponema immobilization test, was blended after addition of motile salmonella, the mixture being gassed with 95% (v/v) $N_2 + 5$% (v/v) CO_2 before and after blending. Treatment for 2 min. decreased the proportion of motile salmonella from *c.* 66 to *c.* 0%, but had no effect on the spirochaetes, *c.* 80% of which continued to show the typical non-translational movements of this organism (Sequeira, 1956). It appeared from these experiments that a blending treatment sufficient to destroy the motility of *Salmonella typhimurium* by deflagellation had little or no effect on the motility of these three types of spirochaete, except when it broke them.

DISCUSSION

Mechanism of deflagellation. Weibull & Tiselius (1945) found that purified flagella of *Salmonella paratyphi* B were very rapidly destroyed by exposure to acid. Our results confirm their further observation that the flagella of live salmonella are equally acid-sensitive. The finding of a very small proportion of normally motile bacteria in suspensions which had received an acid treatment sufficient to destroy all the flagella of the large majority of bacteria was surprising. It seems unlikely that these were bacteria which had escaped contact with the acid, for the bacterial suspension was blown into the buffer solution, and the tube was shaken to ensure good mixing. Weibull & Tiselius (1945) made a somewhat analogous observation: after exposure for 20 min. at pH 3·0 and 20° (at which temperature the destruction of flagella was much slower than at 38°) *c.* 10% of the flagella in a purified preparation were still visible in the electron-microscope, and these persisters were still present even after exposure for 60 min. The temperatures used in our experiments were intended to avoid this heterogeneous behaviour, but it is possible that a lesser degree of such heterogeneity accounts for our results.

The method of rubbing bacteria grown on agar over the surface has not, we think, been previously used for deflagellation. Nicolle (1941, 1943), however, noted that various phages were inactivated by the prolonged rubbing (instead of brief spreading) of suspensions on agar, inactivation being more rapid when a mixture of bacteria and phage was rubbed than when phage alone was rubbed. As to the mechanism of deflagellation by rubbing, it seems *a priori* that bacteria growing on the surface of agar, that is in a film in which any intercellular spaces must be filled with liquid, will be close-packed, each bacterium being in direct contact with its neighbours. When a mass of close-packed bacteria are sheared by rubbing, it seems likely that a flagellum may be caught between two bacterial bodies and so pulled until it breaks. The ease with which bacteria are deflagellated in this way probably accounts for the common statement that bacterial flagella are 'very fragile and easily broken-off'. Our experiments and those described in the literature do not suggest that this could be said of the flagella of bacteria in suspension.

Several authors have reported that flagella may be detached from bacteria by vigorous and prolonged shaking of bacterial suspensions (e.g. de Rossi, 1904, 1905; Craigie, 1931; Gard, 1944), and in some cases it was shown that bacteria after such treatment had lost all or nearly all their flagella. We failed to deflagellate suspensions of *Salmonella typhimurium* by shaking, possibly because the machine used was not sufficiently vigorous. Other authors (Leifson, 1930; Mallett, Koffler & Rinker, 1951) have reported a similar failure. Craigie (1931) stated that even with his specially designed shaker it was possible to deflagellate only when the bacterial suspension was very dense. This suggests that collisions of bacterium with bacterium (or flagellum) are the means by which shaking deflagellates.

High-speed stirring in a blendor was found the most convenient non-lethal method of deflagellation. The way in which the flagella are removed is not clear, except that the equal efficacy of treatment of suspensions of different concentrations establishes that bacterial collisions are not involved, at least when the bacterial concentation is not greater than 5×10^{10} organisms/ml. It seems possible that cavitation is involved. The increased deflagellating effect of treatment at low temperatures, and some incomplete observations which suggest a similar increased efficacy in methyl cellulose solutions, suggest that mere exposure of a flagellated bacterium to a rapid shearing motion in fluid may break off its flagella.

Flagella and bacterial motility. All the experimental data recorded here are compatible with the hypothesis that the motility of *Salmonella typhimurium* and other flagellated bacteria tested results only from the active movement of their flagella, for deflagellation by any of three different methods resulted in loss of motility without loss of viability. Furthermore, when deflagellation was incomplete, and during the recovery of flagellation and motility after blending, the proportions of motile and of flagellated bacteria were approximately equal. By contrast, blending sufficient to deflagellate *S. typhimurium* had little or no effect on the movements of *Treponema pallidum*, *T. duttoni* and *Leptospira canicola*, organisms which lack typical 'extracellular' flagella. These results do not prove that flagella are the locomotor organs of Salmonella; but on the alternative hypothesis (that motility results from alterations of shape of the bacterial body) they can only be accounted for by the assumption that the non-lethal treatment which removed flagella from bacteria of flagellated species also adversely affected their ability to swim by alteration of body shape, even though it did not affect this ability in the tested species of non-flagellated bacteria. It might be argued that various treatments caused both deflagellation and loss of motility because flagella resulted from bacterial movements, instead of being their cause (cf. Pijper, 1946, 1957); the loss of flagella caused by blending of bacteria of the non-motile flagellated *S. typhimurium*, strain SW578, and the subsequent reappearance of flagellated bacteria, cannot easily be reconciled with this view. We may refer here to some earlier observations on this problem. It was reported by de Rossi (1904, 1905) that prolonged vigorous shaking of a dense suspension of a *Bacillus* sp., or of the typhoid bacillus, resulted in loss of motility and in the detachment of the flagella from the bodies. Our experiments are consistent with this. Pijper (1949, 1957) reported that *S. typhi* after shaking for 15 min. no longer showed 'tails' when examined by sunlight dark-ground microscopy, though their motility was unimpaired, and cited this as evidence that amputation of flagella does not affect motility. Mallett, Koffler & Rinker (1951) reported that shaking more vigorous than that used by Pijper did not affect complete deflagellation of *S. typhi*, and it seems probable that the disappearance of 'tails' which he observed resulted either from a decrease in number or length of flagella, or from their diminished aggregation, but not from complete deflagellation.

It was observed in the present work that salmonella with only flagellar stumps (left by incomplete deflagellation) or with the short flagella which first appeared on incubation after complete deflagellation, actively rotated, but showed little or no translational movement. The minimum length of flagellum needed for translational movement was estimated as *c.* 3 μ. If the active movement of a flagellum consists in the continuous propagation of a circular (helical) wave from base to tip, then a certain minimum length may be needed for the generation of the axial thrust which such movement produces, when there is resistance to the torque also produced (Hancock, 1953). In the case of a two-dimensional wave motion a length of at least one wavelength is needed for longitudinal movement (Gray, 1953). The wavelength of the flagella of *Salmonella typhimurium*, strain LT2, was found to be about 2·0 μ.

Growth of flagella. The gradual increase in mean flagellar length observed when deflagellated salmonella were incubated indicates that the flagella of this organism grow gradually, and are not produced by the sudden extrusion of a fully formed flagellum. Analysis of the data on mean no. flagella/bacterium and of mean flagellar length in such experiments suggests, as shown above, that a flagellum increases in length for only a limited period after its inception, about half the flagella present at any one time being 'fully grown' and incapable of regeneration when broken off by trauma. Pedigree experiments have, however, shown that motility can be transmitted unilinearly, i.e. down one line of descent, for 20 or more generations (Stocker, 1956*a*, *b*; Lederberg, 1956), and there is evidence that this results from persistence of a single flagellum (Quadling, 1958). It therefore appears that a flagellum gradually elongates for a limited period (probably between 1 and 2 generation-times), and thereafter persists, and continues to function, for several or many generations, without further elongation. The notion that bacterial flagella grow in length for a limited period only provides a possible explanation for several unpublished observations made in this laboratory. Dr D. Kerridge (personal communication) has found that when bacteria grown in broth are deflagellated and then incubated in a defined medium flagella reappear, but that the average no. flagella/bacterium is always less than before deflagellation, as would be expected if a proportion of the original flagella were 'fully-grown'. Dr C. Quadling (personal communication) has transferred actively growing bacteria from an environment in which flagella developed normally to one in which formation of new flagella ceased, though bacterial growth continued. When such cultures were deflagellated soon after transfer to the new environment some motile bacteria appeared on further incubation, i.e. at least some flagella were regenerated; but when the bacteria were deflagellated after a longer period in the new environment there was no, or very little, regain of motility, presumably because all the flagella initiated before formation of new flagella ceased were 'fully grown' by the time the flagella were removed by blendor treatment. It has also been noted (Stocker & Quadling, unpublished) that in salmonella the mean no. flagella/bacterium may be varied over a wide range by alteration of genotype or of environment but that the length distribution of the flagella is not obviously altered by such changes. It seems that the 'basal granule' presumed to secrete the flagellum has some degree of autonomy, and that once formed it secretes flagellum for a limited time, even though the conditions are such that no new basal granules are initiated. Dr D. Kerridge (personal communication) has indeed shown that under some conditions bacteria can secrete flagella even though they are unable to grow.

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REFERENCES

Beighton, E., Porter, A. M. & Stocker, B. A. D. (1958). X-ray and related studies of the flagella of non-motile bacteria. *Biochim. biophys. Acta*, **29**, 8.

Bradfield, J. R. G. & Cater, D. B. (1952). Electron-microscopic evidence on the structure of spirochaetes. *Nature, Lond.* **169**, 944.

Brinton, C. C., Buzzell, A. & Lauffer, M. A. (1954). Electrophoresis and phage susceptibility studies on a filament-producing variant of the *E. coli* B bacterium. *Biochim. biophys. Acta*, **15**, 533.

Cayton, H. R. & Preston, N. W. (1955). *Spirillum mancuniense* n.sp. *J. gen. Microbiol.* **12**, 519.

Craigie, J. (1931). Studies on the serological reactions of the flagella of *B. typhosus*. *J. Immunol.* **21**, 417.

de Rossi, G. (1904). Sui fenomeni di agglutinazione dei batteri. *Arch. Sci. Med.* **28**, 1.

de Rossi, G. (1905). Sui fenomeni di agglutinazione dei batteri. *Arch. Sci. Med.* **29**, 455.

DUNCAN, J. T. (1935). Inactivation of the 'H' antigen by dilute mineral acid. *Brit. J. exp. Path.* **16**, 405.

GARD, S. (1944). Preparation of bacterial flagella. *Ark. Kemi Min. Geol.* **19** A, no. 21.

GRAY, J. (1953). Undulatory propulsion. *Quart. J. micr. Sci.* **94**, 551.

HANCOCK, G. J. (1953). The self-propulsion of microscopic organisms through liquid. *Proc. Roy. Soc.* A, **217**, 96.

HERSHEY, A. D. & CHASE, M. (1952). Independent function of viral protein and nucleic acid in growth of bacteriophage. *J. gen. Physiol.* **36**, 39.

LEDERBERG, J. (1956). Linear inheritance in transductional clones. *Genetics*, **41**, 845.

LEIFSON, E. (1930). A method of staining bacterial flagella and capsules together with a study of the origin of flagella. *J. Bact.* **20**, 203.

LEIFSON, E. (1951). Staining, shape and arrangement of bacterial flagella. *J. Bact.* **62**, 377.

MALLET, G. E., KOFFLER, H. & RINKER, J. N. (1951). The effect of shaking on bacterial flagella and motility. *J. Bact.* **61**, 703.

MEYNELL, G. G. (1955). Some factors affecting the resistance of mice to oral infections by *Salm. typhimurium*. *Proc. Roy. Soc. Med.* **48**, 916.

NICOLLE, P. (1941). Diminution du nombre des plages bacteriophagiques sur gélose en fonction du temps d'étalement. *C.R. Soc. Biol., Paris*, **135**, 709.

NICOLLE, P. (1943). Appréciation de la taille des corpuscules bactériophages par leur sensibilité au frottement. *Ann. Inst. Pasteur*, **69**, 116.

PIJPER, A. (1946). Shape and motility of bacteria. *J. Path. Bact.* **58**, 525.

PIJPER, A. (1948). Bacterial flagella and motility. *Nature, Lond.* **161**, 200.

PIJPER, A. (1949). Evidence that amputation of bacterial flagella does not affect motility. *Science*, **109**, 379.

PIJPER, A. (1957). Bacterial flagella and motility. *Ergebn. ImmunForsch.* **30**, 37.

QUADLING, C. (1958). The unilinear transmission of motility and its material basis in Salmonella. *J. gen. Microbiol.* **18**, 227.

QUADLING, C. & STOCKER, B. A. D. (1956). An environmentally induced transition from the flagellated to the non-flagellated state in Salmonella: the fate of parental flagella at cell division. *J. gen. Microbiol.* **15**, i.

SEQUEIRA, P. J. L. (1956). The morphology of *Treponema pallidum*. *Lancet*, ii, 749.

STOCKER, B. A. D. (1956*a*). Bacterial flagella: morphology, constitution and inheritance. In *Bacterial Anatomy*. *Symp. Soc. gen. Microbiol.* **6**, 19.

STOCKER, B. A. D. (1956*b*). Abortive transduction of motility; a non-replicated gene transmitted through many generations to a single descendant. *J. gen. Microbiol.* **15**, 575.

STOCKER, B. A. D., ZINDER, N. D. & LEDERBERG, J. (1953). Transduction of flagellar characters in Salmonella. *J. gen. Microbiol.* **9**, 410.

SWAIN, R. H. A. (1955). Electron microscopic studies of the morphology of pathogenic spirochaetes. *J. Path. Bact.* **69**, 117.

WEIBULL, C. (1948). Some chemical and physico-chemical properties of the flagella of *Proteus vulgaris*. *Biochim. biophys. Acta*, **2**, 351.

WEIBULL, C. (1949). Chemical and physico-chemical properties of the flagella of *Proteus vulgaris* and *Bacillus subtilis*. A comparison. *Biochim. biophys. Acta*, **3**, 378.

WEIBULL, C. & TISELIUS, A. (1945). Note on the acid hydrolysis of bacterial flagella. *Ark. Kemi Min. Geol.* **20** B, no. 3.

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McKee, R. K. (1959). *J. gen. Microbiol.* **20**, 686–696

Factors Affecting the Toxicity of Solanine and Related Alkaloids to *Fusarium caeruleum*

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SUMMARY: Solanine toxicity was assessed by its effect on the viability of spores of *Fusarium caeruleum*. Factors which were found to influence toxicity included solanine concentration, period of exposure, hydrogen-ion concentration, spore density, the Na ion/Ca ion ratio and the previous history of the spores. The toxicity of some related compounds was determined, and the toxicity of solanine to some other micro-organisms. The effect of solanine on the growth of *F. caeruleum* in culture was investigated. The nature of the toxic action of solanine on fungus spores and the similarity of spore toxicity to haemolytic activity are discussed. It is concluded that the presence of solanine is unlikely to be an important factor in preventing fungal infection of those tissues in which it is found.

The act of wounding potato tubers stimulates the development of a meristem near the wound and the eventual formation of a suberized wound periderm. This periderm provides the exposed cells with mechanical protection against fungi and bacteria, but it has been shown that the adjacent unsuberized tissues also acquire enhanced resistance to infection (McKee, 1954; Müller, 1957).

The changes in chemical composition which take place in the tissues responding to the wound stimulus include a marked increase in their content of solanine (McKee, 1955), a surface-active alkaloid which at low concentrations lyses red blood corpuscles and which is toxic to fungi (Schmidt, 1933). This paper describes observations on the toxicity, *in vitro*, of solanine and related alkaloids to *Fusarium caeruleum* (Lib.) Sacc., the cause of dry rot of potatoes; the observations were made during an investigation on the relation between solanine content and tissue resistance (McKee, 1956). The action of solanine on some other micro-organisms is also recorded. Some of the results included here were reported previously in brief (McKee, 1955, 1957).

METHODS

Alkaloids. Samples of solanine (α-solanine, Kuhn & Löw, 1954), demissine and tomatine were isolated, respectively, from potato sprouts and from young leaves of *Solanum demissum* and *Lycopersicon pimpinellifolium*, by methods based on that described by Wolf & Duggar (1946). α-Chaconine (Kuhn & Löw, 1954), another trisaccharide of solanidine which occurs in about the same concentration as solanine in potato tissues, was separated by chromatography on buffered cellulose columns (McKee, 1956). Solanidine was prepared by the hydrolysis of solanine (Arnold, 1950). The alkaloids were recrystallized and their purity examined by paper chromatography (McKee, 1957). The relationships and composition of the *Solanum* glyco-alkaloids were summarized by Schreiber (1958).

Organisms. An isolate of *Fusarium caeruleum* (Lib.) Sacc. from a dry-rot lesion in a potato tuber was maintained on potato glucose agar during the investigation. Subcultures were grown from single spores to reduce the risk of selecting fast-growing variants; the appearance of the isolate remained unchanged throughout.

Specimens of *Phytophthora infestans*, *Fusarium avenaceum*, *F. culmorum*, *Streptomyces scabies*, *Erwinia* spp. and *Pseudomonas* spp. were also isolated from diseased potato tubers. Cultures of *Bacillus subtilis* and *Micrococcus luteus* were obtained from the culture collection of the Department of Microbiology, Nottingham University School of Agriculture.

Effect of alkaloids on viability of spores. Solutions were prepared containing the alkaloids (as acetates) and buffer and such other salts as are indicated in the description of the experimental work. Phosphate buffers (M/15) were used except where the effect of calcium ion was being investigated, when veronal buffer was used. All experiments were carried out at room temperature (*c.* 18°) and at pH 7·6 unless otherwise stated. Spores were placed in small volumes (usually 0·1 ml.) of the alkaloid solution and after an interval of 2–3 hr. sample loopfuls of the suspension were streaked on to plates of water agar acidified to pH 3·5 with lactic acid. The % germination was determined *c.* 16 hr. later, each estimate being based on at least 50 spores. Abbott's correction for natural mortality was applied but this was often unnecessary as germination in control solutions without alkaloid was usually *c.* 98 %. The % kill figures were changed into probits (Finney, 1952) and were plotted against the dose, transformed logarithmically (dosage) to give a linear relationship between the two.

Effect of alkaloids on rate of growth of Fusarium caeruleum. Growth was estimated by the rate of increase in diameter of colonies on potato extract agar in Petri dishes as basal medium, to which the alkaloids were added.

RESULTS

Effect of solanine on fungus spores

Microscopic examination of spores of *Fusarium caeruleum* which had been exposed to toxic concentrations of solanine showed that the affected cells were shrunken and that their contents were dense and uniform in contrast to the vacuolate appearance of the unaffected cells. The four to five cells in each spore reacted individually, any which were unchanged in appearance retaining the ability to germinate. Spores of *F. avenaceum* and *F. culmorum* behaved similarly. Zoospores of *Phytophthora infestans*, which consist of naked protoplasts in the presence of solanine came to rest prematurely, suddenly increased in size greatly and then disintegrated, the contents being dispersed—a response analogous to that of the red blood corpuscle.

Relation between concentration of solanine and its toxicity to spores

The results of a typical experiment with *Fusarium caeruleum* spores are shown in Fig. 1 where the probits of % inhibition of germination are plotted against dose of solanine. The linear relationship was found to hold over wide limits of germination, facilitating the calculation of LD 50 values (probit = 5). These were determined at pH 7·6 unless otherwise stated.

Deviations from the line of best fit were small within individual experiments but more variation was found between experiments carried out at different times. Thus repetition, at intervals, of the experiment recorded in Fig. 1 gave LD 50 values in the range 20–36 mg. solanine/l. with a mean value of 33 mg./l. The mean value of the regression coefficient of probit on dosage was 8·8, a high value which indicates that small changes in solanine concentration greatly influenced the proportion of spores surviving.

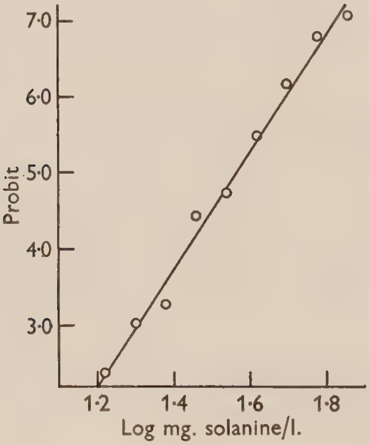

Fig. 1

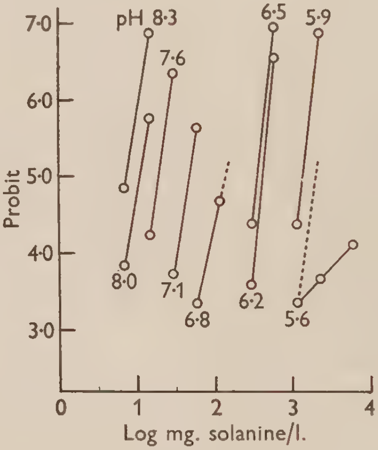

Fig. 2

Fig. 1. Dosage-response curve for inhibition of germination of spores of *Fusarium caeruleum* by solanine.

Fig. 2. Dosage-response curves at different pH values for inhibition of germination of spores of *Fusarium caeruleum* by solanine.

Effect of period of exposure of spores to solanine

Spore suspensions were mixed with solanine solutions and samples withdrawn immediately and at intervals in order to determine the rate of action of solanine (Table 1). No germination took place in the test solutions as *Fusarium caeruleum* spores remain dormant when submerged unless very near the surface. The time required for maximal response proved short at the higher solanine concentrations, while even at the lowest concentration used no further decrease in viability was found for periods of exposure greater than 1 hr.

Table 1. *Percentage germination of* Fusarium caeruleum *spores after exposure to solanine solutions*

Period of exposure (hr. min.)	Solanine concentration (g./l.) 200	100	50	25
	% germination			
0 0	96	98	99	98
0 1	60	94	.	.
0 2	1	54	94	.
0 4	0	2	87	99
0 8	.	0	4	96
0 15	.	.	0	53
0 30	.	.	.	22
1 0	.	.	.	16
4 0	.	.	.	18
6 0	.	.	.	19
24 0	.	.	.	17

Effect of hydrogen-ion concentration

The haemolytic activity of solanine has been shown to be greater in alkaline than in acid solution (Fischer, 1927) and the effect of hydrogen-ion concentration on toxicity to spores was therefore investigated. The LD50 values at different pH values were determined graphically as indicated in Fig. 2; it will be noted that the slope of the curves was similar except at the lowest pH value where high concentrations of solanine were required. Here, excess acetic acid in the solanine solution may have lowered the pH value below the nominal value; extrapolation parallel to the other curves was made from the point for the lowest solanine concentration but this particular LD50 estimate must be regarded as tentative.

The proportion of solanine which is present as undissociated base at any particular hydrogen-ion concentration may be calculated by using the estimate of the dissociation constant of solanidine, $pK_b = 5{\cdot}38$, given by Bloom & Briggs (1952). In Fig. 3, the LD50 values determined experimentally are plotted against pH values. The theoretical curve based on the assumption that only the undissociated molecules are toxic is also shown; this was adjusted to coincide with the experimental curve at pH 7·0. The good agreement between the two suggests that the undissociated base was in fact responsible for most of the toxicity.

Effect of concentration of spore suspension

The relationship between concentration of spore suspension and solanine toxicity was investigated at pH values 6·5 and 8·0. Spores of *Fusarium caeruleum* were washed with the appropriate buffer solution, separated by centrifugation and made up into strong suspensions from which a series of dilutions in buffer were prepared. Samples from these were mixed with buffer solutions, each containing solanine at a concentration sufficient to allow about 4% germination at the lowest concentration of spores. Samples were plated out after exposure for 4 hr. and % germination observed (Fig. 4). The highest concentration of spores in the test solution was c. 3% by volume. At the higher solanine concentration, increases in spore concentration up to about 40 times the minimum had little effect on % spores affected, though this decreased when the concentration of spores was further increased. At the lower solanine concentration, any increase in spore concentration decreased the kill.

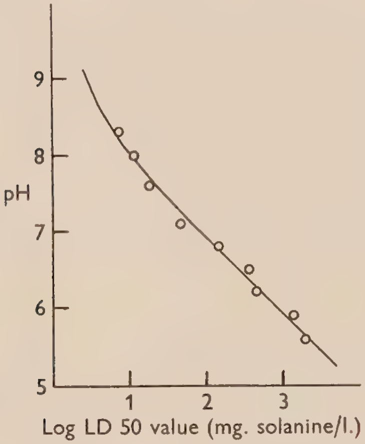

Fig. 3

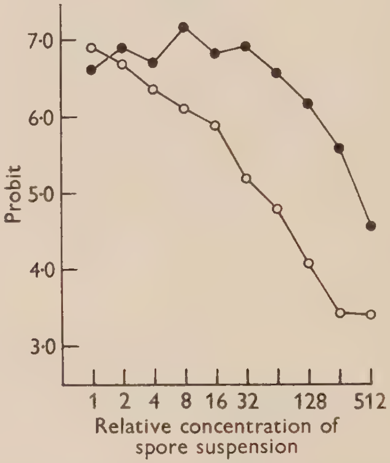

Fig. 4

Fig. 3. Relationship between LD 50 value and pH value for inhibition of germination of spores of *Fusarium caeruleum* by solanine. ○, experimental values; ——, theoretical curve.

Fig. 4. Relationship between response to solanine and concentration of spore suspension (*F. caeruleum*). ○——○, 27 mg. solanine/l., pH 8·0; ●——●, 1110 mg. solanine/l., pH 6·5.

Effect of electrolytes

The activity of many haemolytic substances appears to be influenced by changes in the concentration and nature of the accompanying electrolytes in the test solution (Ponder, 1948) but such changes can themselves cause haemolysis of the red blood corpuscles. The spores of *Fusarium caeruleum* proved to be unaffected by a wide range of salt contents so that it was possible to determine the effect of electrolytes on the toxicity of solanine without having to make allowance for a toxic effect of the salts themselves.

Solanine toxicity was found to increase with increasing concentrations of sodium or potassium ions. Where the concentration of such ions was low, spores were unaffected by exposure even to high solanine concentrations. Addition of glucose up to a concentration of 10% (w/v) did not restore solanine toxicity in such solutions, indicating that their osmotic pressure was not a decisive factor. Preliminary experiments showed that sodium and potassium ions had equivalent effects, so more detailed experiments were carried out with sodium alone (Fig. 5).

Solanine toxicity was decreased in the presence of calcium ions, being dependent on the sodium ion/calcium ion ratio (Fig. 6). An increase in calcium-ion concentration compensated a much larger increase in sodium-ion concentration, the value of the ratio being about 1:350, though this varied with changes in actual ionic concentration (Fig. 7). The effect of calcium was nullified when sufficient EDTA (disodium salt of ethylenediaminetetra-acetic acid) was added to chelate the calcium ions present, these concentrations of EDTA alone being non-toxic to the spores. Solanine toxicity was not diminished in the presence of magnesium ions.

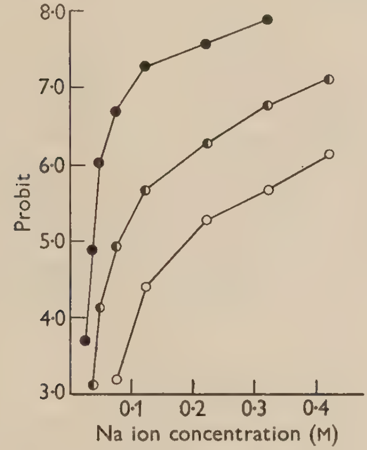

Fig. 5

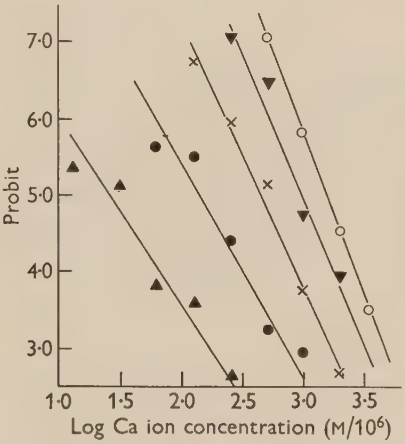

Fig. 6

Fig. 5. Relationship between response of spores of *Fusarium caeruleum* to solanine and concentration of sodium ions. ○——○, 25 mg. solanine/l.; ◐——◐, 42 mg. solanine/l.; ●——●, 62 mg. solanine/l.

Fig. 6. Relationship between response of spores of *Fusarium caeruleum* to solanine and concentration of calcium ions. ▲——▲, 0·07 M-Na^+; ●——●, 0·12 M-Na^+; ×——×, 0·22 M-Na^+; ▼——▼, 0·32 M-Na^+; ○——○, 0·42 M-Na^+. 62 mg. solanine/l.

Sensitivity of spores of Fusarium caeruleum

The LD values of different batches of spores from a stable isolate of *Fusarium caeruleum*, assessed under identical conditions, were found to be influenced by characteristics of the spores themselves. Macrospores from mature cultures were more tolerant of solanine than those from young cultures. A further increase in tolerance occurred in old cultures where individual cells of some of the macrospores developed into thick-walled chlamydospores. These proved. capable of germination in solutions containing solanine in concentrations sufficient to kill all the accompanying macrospore cells—possibly owing to absorption of solanine by the macrospores before the germ-tube had emerged from the chlamydospore.

The sensitivity of spores from cultures grown on potato glucose agar to which 0·4% (w/v) calcium chloride had been added was identical with that of spores produced on potato glucose agar alone. The sensitivity was, however, decreased when 500 mg. solanine/l. was included in the medium as well as the calcium chloride. In a typical experiment, an LD50 value of 68 ± 4 mg.

solanine/l. was obtained for spores from a treated culture, compared with 38 ± 3 mg./l. for spores from the control culture containing calcium chloride but no solanine. The values of the regression coefficient of probit on dosage were always lower for the treated spores than for the controls, but the differences were not significant owing to high values of χ^2 in the probit analysis. The growth rate of *F. caeruleum* was unaffected by solanine in the presence of calcium so it appears unlikely that the decrease in sensitivity can be attributed to the selection of more resistant lines.

Toxicity of solanine to other micro-organisms

Macrospores of *Fusarium avenaceum* and *F. culmorum*, and sporangia of *Phytophthora infestans* were found to have a sensitivity to solanine similar to that of *F. caeruleum* spores, namely, an LD 50 value of *c.* 30 mg. solanine/l. Zoospores of *P. infestans* proved very sensitive, all in a sample being killed by 5 mg. solanine/l. so that the LD 50 value would be even less than this.

The viability of spores of *Streptomyces scabies* was not affected by several hours of exposure to 2000 mg. solanine/l. Bacteria from young cultures of *Bacillus subtilis, Micrococcus luteus, Erwinia* spp. and *Pseudomonas* spp. were also unaffected by this concentration of solanine.

Toxicity of compounds related to solanine

The LD 50 value of α-chaconine was 11 mg./l., compared to 20 mg. solanine/l. for the same sample of spores of *Fusarium caeruleum.* Solanidine (LD 50 value 100 mg./l.) proved less toxic than solanine; this may have been partly due to its low solubility. The undissociated base of solanidine is much less soluble than that of solanine though the solubility of the latter is only 20 mg./l. (Rooke, Bushill, Lampitt & Jackson, 1943). The proportion of *F. caeruleum* spores affected by solanidine after 24 hr. was not greater than that after exposure for 4 hr.

Table 2. LD 50 *values (mg./l.) for solanine, demissine, tomatine and digitonin for* Fusarium caeruleum *spores*

pH value	LD 50 (mg./l.)			
	Solanine	Demissine	Tomatine	Digitonin
5·0	.	.	.	27
5·6	2000	.	460	.
5·9	1100	260	220	.
6·5	260	72	64	18
7·0	85	22	32	.
7·6	20	11	18	.
8·0	11	.	.	14
8·3	8	8	7	.

Demissine and tomatine were more toxic for *Fusarium caeruleum* spores than was solanine, especially in the more acid solutions (Table 2). The free base of both compounds tended to separate as a gel more readily than that of solanine, indicating a low solubility. In contrast to solanidine, the proportion of spores affected increased when the period of exposure was extended to more than 4 hr.

The steroid saponins are closely related in structure to the *Solanum* glycoalkaloids but differ from them in being neutral compounds. The N atom which in the alkaloids forms a secondary or tertiary amine is replaced in the saponins by an O atom in a heterocyclic ring. The LD 50 value of the steroid saponin digitonin showed only minor change over a wide range of pH values (Table 2). The triterpenoid saponins possess haemolytic activity though less so than the steroid saponins (Pethica & Schulman, 1953). 'Saponin, pure white' at 1000 mg./l. showed no toxicity to spores of *F. caeruleum* at pH 4·5 or at pH 7·6.

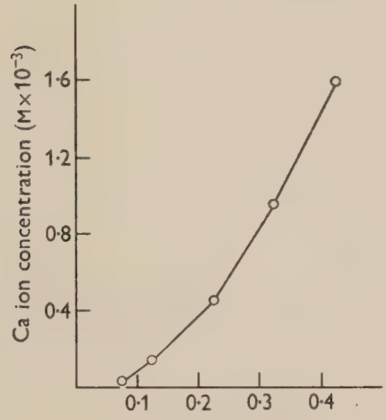

Fig. 7

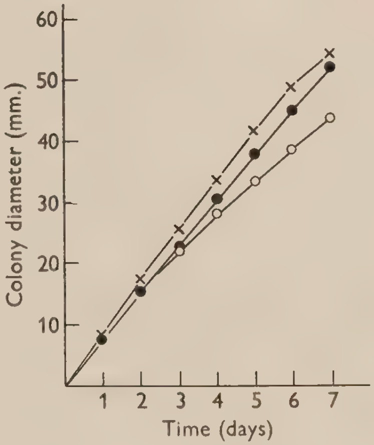

Fig. 8

Fig. 7. **Relationship between concentrations of sodium ion and of calcium ion giving 50 % inhibition of germination of spores of *Fusarium caeruleum* by 62 mg. solanine/l.**

Fig. 8. **Effect of solanine on growth of *Fusarium caeruleum* on potato extract agar. ●——●, potato extract alone; ○——○, potato extract + 500 mg. solanine/l.; ×——×, potato extract + 500 mg. solanine/l. + 1 % (w/v) $CaCl_2$.**

Effect of solanine on the growth of Fusarium caeruleum *in culture*

Potato extract was used as the basal medium in experiments on the effect of solanine on the growth of *F. caeruleum* in culture since the changes in pH. value during growth in this medium were similar to those in tuber tissues attacked by the fungus. The initial growth rate on potato extract containing 500 mg. solanine/l. was identical with that on the extract alone (Fig. 8), but after 3 days the growth rate in the presence of solanine decreased though the fungus continued to grow until the experiment was concluded. Addition of calcium chloride to the medium prevented the depressive action of solanine and also increased the steady growth rate over that of the control. In similar experiments it was shown that the retarding action of solanine was prevented when 2 % (w/v) glucose was added to the potato-extract medium.

The changes in pH value (Table 3) which took place during growth of *Fusarium caeruleum* on potato-extract liquid medium offer an explanation of the delayed effect of solanine on growth rate and of the effect of added glucose. In the medium alone, the pH value increased until, after several days, a value was reached where solanine was highly toxic; with added glucose the pH value decreased for several days and then began to increase. The fact that growth continued, though at a lower rate, in the presence of solanine even when the medium had become alkaline might be taken to indicate that the growing mycelium was less sensitive (or had become less sensitive) to solanine than the macrospores. However, solanine was found to be hydrolysed (Table 4) by the fungus to the less toxic solanidine via the intermediate glycosides, whose toxicity was not assessed as samples of them were not isolated. It is therefore difficult to define the conditions to which the advancing mycelium was, in fact, exposed.

Table 3. *Change in* pH *value of culture media during growth of* Fusarium caeruleum

Period of growth (days)	Potato-extract medium	Potato-extract medium + 2 % (w/v) glucose
0	5·2	5·2
1	5·8	5·4
2	5·8	5·3
3	6·7	5·0
4	7·4	4·5
5	8·2	3·8
8	8·6	7·6
14	8·7	7·6

Table 4. *Hydrolysis of solanine during growth of* Fusarium caeruleum *in potato-extract medium containing initially solanine* (500 *mg./l.*) *and calcium chloride* (1 %, *w/v*)

Period of growth (days)	Hydrolysis products detected chromatographically			
	α-Solanine	β-Solanine	λ-Solanine	Solanidine
0	+	−	−	−
3	+	+	+	−
5	−	−	+	(+)
7	−	−	+	+
10	−	−	+	+
16	−	−	(+)	+

+, Present; −, absent; (+), trace.

DISCUSSION

The rapid disintegration of the naked protoplasts of the zoospores of *Phytophthora infestans* when exposed to toxic concentrations of solanine suggests that the alkaloid affects the surface membrane of the spore, and that the toxic action of solanine on fungal cells is thus similar in nature to its haemolytic activity. The haemolytic activity of some surface active agents has been shown to be greater than would be expected from their surface activity alone, and to be related to their ability to penetrate and cause collapse of cholesterol monolayers which may be considered as experimental analogues of the cholesterol-phospholipid-lipoprotein complex of the cell envelope (Schulman, Pethica, Few & Salton, 1955). The molecule of the aglycone solanidine can be derived structurally from that of cholesterol by the addition of a nitrogen atom; the toxicity of the glycoside solanine and related compounds may well be connected with this relationship. The lack of toxicity shown by the surface-active triterpenoid saponin to *Fusarium caeruleum* is of interest in this respect as is the absence of toxicity of solanine for bacteria.

The relationship between solanine toxicity and hydrogen-ion concentration indicates that toxicity is restricted to molecules of undissociated base whose concentration must exceed a certain value before the surface membrane is affected. Equilibrium is maintained by association of ionized molecules in the solution when the undissociated base is removed by combination with spores. Thus the concentration of the spore suspension had more effect on solanine toxicity at pH 8·0 than at pH 6·5 where most of the solanine was present in the ionized state. The dependence of solanine toxicity, or perhaps of spore sensitivity, on the relative concentrations of sodium or potassium and calcium ions is striking, and it would be of interest to see whether the haemolytic activity of solanine is similarly affected.

The part played by the solanine in potato tissues in protecting them against fungal infection and subsequent invasion is difficult to assess since solanine toxicity *in vitro* is influenced so much by factors, such as pH value, which may vary in the tissues concerned. However, it would seem unlikely that the solanine content plays a decisive part in controlling infection, at least by *Fusarium caeruleum*, in view of the ability of this fungus to grow in the presence of solanine in media where conditions appear similar to those found in dry-rot lesions in tuber tissues. This conclusion is supported, though negatively, by the fact that no correlation was demonstrated between susceptibility of tubers to dry-rot infection and their ability to accumulate solanine near wounds when these were measured at intervals during the storage season (McKee, 1956).

I would like to thank Professor C. G. C. Chesters and Dr A. R. Wilson for their interest and encouragement in this work which was carried out at the Agricultural Research Council Potato Storage Investigation, c/o University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire.

REFERENCES

Arnold, W. (1950). Eine verbesserte Methode zur Gewinnung und quantitativen Bestimmung des Solanins, des Glycoalkaloids der Kartoffelpflanze. *Pharmazie*, 5, 490.

Bloom, H. & Briggs, L. F. (1952). Solanum alkaloids. Part. IX. The relation between constitution and basicity in solasodine, its derivatives and solanidine. *J. chem. Soc.* 3591.

Finney, D. J. (1952). *Probit Analysis.* Cambridge University Press.

Fischer, R. (1927). Der Einfluss der Wasserstoffionenkonzentration auf die Solanin-Hämolyse. *Biochem. Z.* **209**, 319.

Kuhn, R. & Löw, I. (1954). Die Konstitution des Solanins. *Angew. Chem.* **66**, 639.

McKee, R. K. (1954). Dry-rot disease of the potato. VIII. A study of the pathogenicity of *Fusarium caeruleum* (Lib.) Sacc. and *Fusarium avenaceum* (Fr.) Sacc. *Ann. appl. Biol.* **41**, 417.

McKee, R. K. (1955). Host-parasite relationships in the dry-rot disease of potatoes. *Ann. appl. Biol.* **43**, 147.

McKee, R. K. (1956). *Studies on the toxicity of solanine and related compounds to micro-organisms and on the influence of solanine content on the resistance of potato tuber tissues to fungal infection.* University of Nottingham, Ph.D. Thesis.

McKee, R. K. (1957). Solanine and related alkaloids. *Nature, Lond.* **179**, 313.

Müller, K. O. (1957). Wound healing of the potato tuber in relation to infection by *Phytophthora infestans* (Mont.) de Bary. *Aust. J. Biol. Sci.* **10**, 189.

Pethica, B. A. & Schulman, J. H. (1953). The physical chemistry of surface active agents. *Biochem. J.* **53**, 177.

Ponder, E. (1948). *Hemolysis and Related Phenomena.* London: J. and A. Churchill.

Rooke, H. S., Bushill, L. H., Lampitt, L. H. & Jackson, E. M. (1943). Solanine, glycoside of the potato. I. Its isolation and determination. *J. Soc. chem. Ind., Lond.* **62**, 20.

Schmidt, M. (1933). Zur Entwicklungsphysiologie von *Cladosporium fulvum* und über die Widerstandfähigkeit von *Solanum racemigerum* gegen diesen Parasiten. *Planta*, **20**, 407.

Schreiber, K. (1958). Über einige Inhaltsstoffe der Solanaceen und ihre Bedeutung für die Kartoffelkäferresistenz. *Ent. exp. & appl.* **1**, 28.

Schulman, J. H., Pethica, B. A., Few, A. V. & Salton, M. R. J. (1955). The physical chemistry of haemolysis and bacteriolysis by surface active agents and antibiotics. *Progr. Biophys.* **5**, 41.

Wolf, M. J. & Duggar, B. M. (1946). Estimation and physiological role of solanine in the potato. *J. Agric. Res.* **73**, 1.

(*Received* 8 *December* 1958)

Lev, M. (1959). *J. gen. Microbiol.* **20**, 697–703

The Growth-Promoting Activity of Compounds of the Vitamin K Group and Analogues for a Rumen Strain of *Fusiformis nigrescens*

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SUMMARY: With a strain of *Fusiformis nigrescens* as test organism, the potencies of compounds of the vitamin K group were estimated, and the results compared with those from animal assay techniques. The compounds showed in the main the same relative activity for the micro-organism as for higher animals, with two exceptions: 1:4-naphthoquinone was highly active for *F. nigrescens* but is inactive for animals; phthiocol, which is active for animals, inhibited the response of *F. nigrescens* to menaphthone, i.e. phthiocol acted as an anti-vitamin K for the micro-organism. Most of the compounds tested were toxic in the range 1–5 μg./ml.; vitamins K_1, K_2 and a water soluble form of menaphthone (menaphthone bisulphite) were relatively less toxic.

The synthesis of vitamin K by certain bacteria is well known, and Woolley & McCarter (1940) reported a stimulation of the growth of *Mycobacterium paratuberculosis* (Johne's bacillus) by menaphthone and phthiocol. However, subcultures of this organism in the basal medium supplemented by the vitamin did not always show growth, and Glavind & Dam (1948), using another strain of *M. paratuberculosis*, were unable to confirm the results of Woolley & McCarter. Large amounts of synthetic forms of vitamin K are toxic for micro-organisms (Armstrong, Spink & Kahnke, 1943). This non-specific toxicity led Almquist (1954) to doubt the feasibility of a microbiological assay for vitamin K. However, the requirement for vitamin K shown by a rumen strain of *Fusiformis nigrescens* (Lev, 1958) suggested the use of this organism to estimate the relative potencies of analogues of this vitamin.

METHODS

Organism. A strain of *Fusiformis nigrescens* isolated from bovine rumen contents (Lev, 1958) was used. It is a strict anaerobe, and requires blood in addition to vitamin K. The medium used in the assay procedure was meat extract broth (MEB), which consisted of: meat extract (Difco) 3 g.; NaCl 5 g.; peptone (Difco) 10 g.; distilled water to 1 l. The medium was adjusted to pH 7·5, distributed in 10 ml. amounts, and autoclaved for 15 min. at 15 lb./sq.in.

Human blood was used in the majority of experiments; horse blood was also used. The blood was centrifuged slowly. A 1/35 dilution of red blood cells was made in distilled water and 0·1 ml. was added to each tube of MEB. This dilution of laked blood, with vitamin K, allowed good growth of the organism without interfering with optical density readings.

The compounds to be assayed were made up as 10 mg./ml. solutions in absolute ethanol and subsequent dilutions, which were either solutions or emulsions, were made in distilled water. A typical assay procedure was as follows. Tubes of MEB were heated in a boiling water bath for 10 min., cooled and 0·1 ml. of the diluted laked red cells added. Dilutions of vitamin K to give final concentrations of 0·05–10 μg./ml. were added and the tubes were inoculated with 0·05 ml. of a two-day culture of *Fusiformis nigrescens* in MEB + laked red cells + 0·5 μg. menaphthone/ml. Control tubes included in each assay consisted of MEB + red cells. The tubes were placed in an anaerobic jar and incubated at 39° in an atmosphere of 90% (v/v) H_2 + 10% (v/v) CO_2. After incubation for 2 days, optical density readings were taken at 520 mμ with a Beckman spectrophotometer. In one experiment readings were taken with a Hilger Spekker absorptiometer using a no. 7 filter.

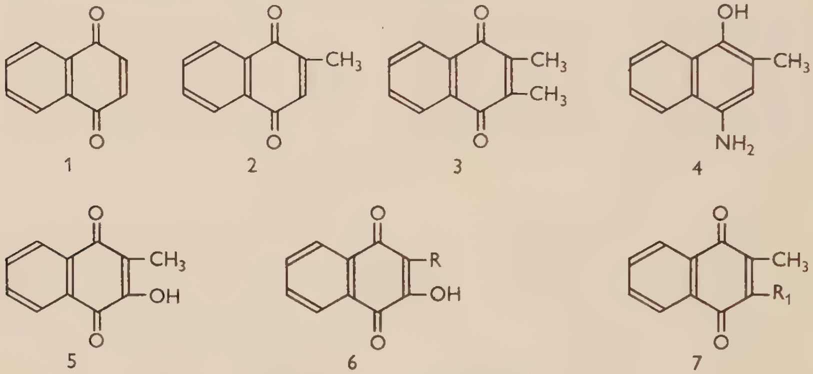
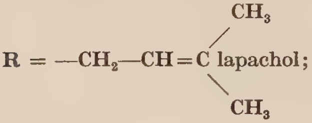


Fig. 1. Analogues etc. of vitamin K group tested.

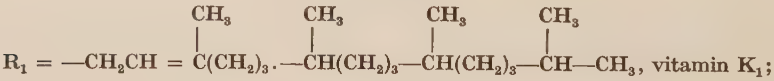
(1) 1:4-naphthoquinone; (2) menaphthone; (3) 2:3-dimethylnaphthoquinone; (4) vitamin K_5; (5) phthiocol;

(6) 

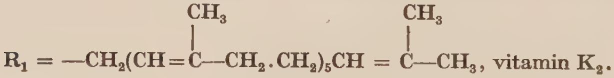
$R = -CH_2-CH=C\genfrac{}{}{0pt}{}{\diagup CH_3}{\diagdown CH_3}$ lapachol;

$R = -(CH_2)_9-C_6H_{11}$ (cyclohexyl) M1953;

$R = -(CH_2)_3-C_6H_{11}$ (cyclohexyl) M1916;

(7) 

$R_1 = -CH_2CH = \overset{CH_3}{\overset{|}{C}}(CH_2)_3.-\overset{CH_3}{\overset{|}{C}}H(CH_2)_3-\overset{CH_3}{\overset{|}{C}}H(CH_2)_3-\overset{CH_3}{\overset{|}{C}}H-CH_3$, vitamin K_1;



$R_1 = -CH_2(CH=\overset{CH_3}{\overset{|}{C}}-CH_2.CH_2)_5CH = \overset{CH_3}{\overset{|}{C}}-CH_3$, vitamin K_2.

The structures of the compounds tested are shown in Fig. 1.

RESULTS

A characteristic of all assays was the toxicity of the used compounds, beyond a critical concentration. Compounds generally became toxic at concentrations greater than those at which maximum growth occurred (Figs. 2–6) and is indicated where the graphs end. Vitamins K_1 and K_2 were relatively non-toxic (Fig. 2) and the water soluble form of menaphthone (menaphthone bisulphite) was less toxic than the fat soluble menaphthone, i.e. in assays menaphthone was toxic in amounts greater than 1 µg./ml., whereas the bisulphite compound was not toxic at 10 µg./ml.

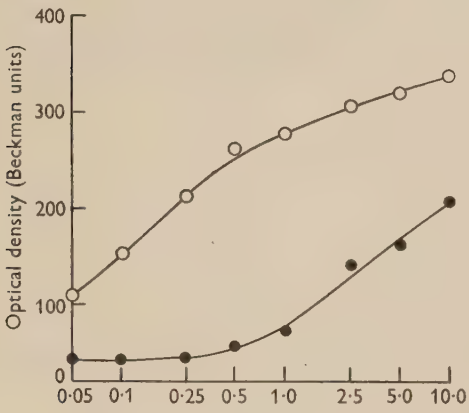


Fig. 2

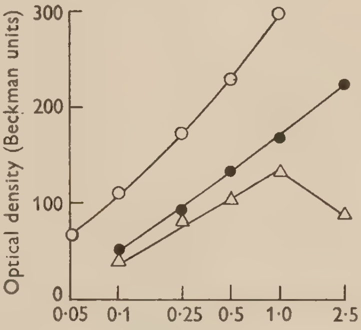


Fig. 3

Fig. 2. Comparative activities of vitamins K_1 (○) and K_2 (●).

Fig. 3. Comparative activities of (I) 1:4-naphthoquinone (○), (II) menaphthone (●) and (III) 2:3 dimethyl 1:4 naphthoquinone (△).

The results of the comparative activity of vitamin K-like compounds for *Fusiformis nigrescens* are compared with their reported activities for animals in Table 1.

Assays of 1:4-naphthoquinone, 2-methyl (menaphthone) and 2:3-dimethyl derivatives are shown in Fig. 3. 1:4-Naphthoquinone was more effective than

Table 1. *Comparative activity of the vitamin K group compounds and analogues for animals and for* Fusiformis nigrescens

Compound	Activity for *F. nigrescens*	Activity for animals	Reference
1:4-Naphthoquinone	++++	0	Fieser, Tishler & Sampson (1941)
Menaphthone	++++	++++	
2:3-Dimethyl-1:4-naphthoquinone	+	+	
Vitamin K_1	+++	+++	
Vitamin K_2	+	+++	
Vitamin K_5	+++	+++	
Phthiocol	–	+++	
Lapachol	++*	0	
M 1916	++*	–	Smith, Fradkin & Lackey (1946)
M 1953	++*	?	

++++, High activity (= menaphthone); –, anti-vitamin K activity; 0, no activity; ?, activity not known.

* Stimulatory at low concentrations.

menaphthone, which had greater activity than the 2:3-dimethyl derivative; 1:2-naphthoquinone showed slight activity. Menaphthone appears to be 1·4–2·5 times as active as vitamin K_1 when equal weights of the two compounds are used. When compared on an equimolar basis vitamin K_1 was 1·6 times more potent than menaphthone. Vitamin K_2 showed less than one-tenth of the activity of vitamin K_1 in promoting the growth of *Fusiformis nigrescens*. Phthiocol was inactive; this analogue was reported (Fieser, Tishler & Sampson, 1941) to have vitamin K activity in animals, but less than menaphthone. It was originally isolated from *Mycobacterium tuberculosis*, probably as a degradation product of vitamin K_1 (Karrer, 1950). However, as can be seen from Fig. 4, it has no vitamin K activity for *F. nigrescens*. The structural similarity of phthiocol to menaphthone, together with the lack of a growth-promoting activity, suggested that this substance may act as an anti-vitamin for *F. nigrescens*. This was shown (Fig. 4) when a constant amount of phthiocol, to give a final concentration of 0·5 μg./ml., was added to a range of menaphthone concentrations. Stimulation of growth occurred when the amount of menaphthone was greater than that of phthiocol. No attempt was made to determine whether this inhibition was competitive.

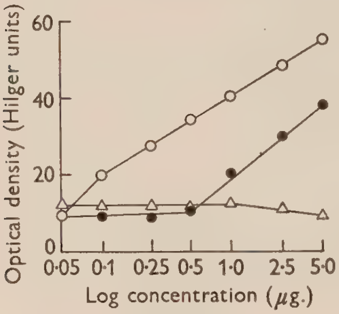


Fig. 4

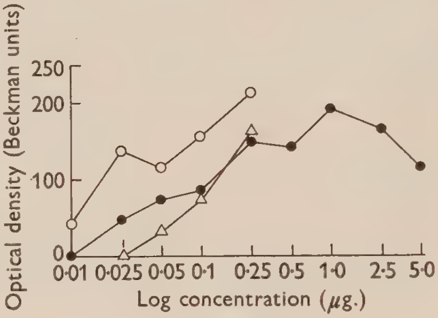


Fig. 5

Fig. 4. Inhibition by phthiocol of growth response of *F. nigrescens* to menaphthone. ○, Menaphthone; ●, menaphthone + 0·5 μg./ml. phthiocol; △, phthiocol.

Fig. 5. Comparative activities of M 1953 (○), M 1916 (△) and lapachol (●).

The derivatives of phthiocol tested, compounds M1953, M1916 and lapachol (Fig. 5), showed high activity at low concentrations, but became toxic at relatively low levels. Compound M1916 has haemorrhagic properties in rats (Smith, Fradkin & Lackey, 1946). Because of this growth-stimulating power of an anti-vitamin K compound for *Fusiformis nigrescens*, it was of interest to determine whether other compounds with known anti-vitamin K activity could behave in a similar manner. Dicoumarol & Warfarin were investigated, but did not stimulate *F. nigrescens*. In another series of experiments (Lev, 1958), dicoumarol was found to inhibit competitively the response of *F. nigrescens* to menaphthone.

Vitamin K_5 (4-amino- 2-methyl- 1-naphthol) had activity for *Fusiformis nigrescens*, although it was not as active as menaphthone (Fig. 6). Richert

(1944) showed that the chick converts vitamin K_5 to menaphthone, and it seems possible that this conversion may also be effected by *F. nigrescens.*

Several reports, e.g. Prosperi & Lottini (1949), have suggested an interaction between vitamin E and vitamin K. An experiment was made to determine whether vitamin E (DL-α-tocopherol) stimulated *Fusiformis nigrescens* and also whether any interaction occurred between α-tocopherol and vitamin K_1. The results were negative; α-tocopherol neither stimulated the growth of *F. nigrescens,* nor had it any effect on the response of this organism to vitamin K_1. In this experiment, because of the opacity of the suspension of α-tocopherol in the medium, additional controls consisting of dilutions not inoculated with the test organism were made.

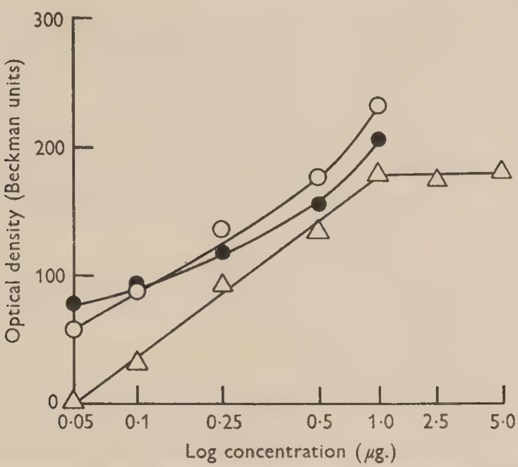


Fig. 6. Comparative activities of menaphthone (○) and vitamins K_5 (●) and K_1 (△) on growth response of *Fusiformis nigrescens.*

DISCUSSION

The results presented here suggest a useful microbiological method for estimating vitamin K and the potencies of the compounds tested follow in the main the same order as that found by animal assay techniques. However, the specificity of *Fusiformis nigrescens* for the vitamin K group differs in two major respects from that of animals; (*a*) 1:4-naphthoquinone is highly active for the micro-organism but shows no activity in the chick assay; (*b*) phthiocol, which shows appreciable vitamin K activity for animals, antagonizes the growth stimulation by menaphthone of *F. nigrescens.* The micro-organism thus shows a greater specificity for vitamin K with respect to phthiocol than higher animals, which is the reverse of what is commonly found in microbiological assays.

Vitamin K_1 was found by Almquist & Klose (1940) to be 1·25 to 1·5 times as potent as vitamin K_2 when compared on a molar basis. The difference between the chick and microbiological assays may be due to the greater insolubility of vitamin K_2 and the subsequent difficulty in absorption of the vitamin by the micro-organism. Compound M1916, which shows haemorrhagic (anti-vitamin K) properties when given to rats, has a high vitamin K activity for *Fusiformis nigrescens* at very low concentrations. This does not necessarily indicate a difference in specificity between *F. nigrescens* and rats for compound M1916, as rats fed this compound and a vitamin K-deficient diet were not included in the experiments of Smith *et al.* (1946).

The toxicity to *Fusiformis nigrescens* of many of the compounds tested in concentrations greater than 1–5 μg./ml. is of interest. Vitamin K_1 is relatively non-toxic, as would be expected, because it is present in large amounts in the grass content of the bovine rumen, from which this strain of *F. nigrescens* was originally isolated, and may also be, as Fieser, Tishler & Sampson (1941) suggested, the complete form of the vitamin which is synthesized from menaphthone. This non-toxic character of vitamin K_1 compared with the synthetic forms of the vitamin for *F. nigrescens* can be seen in the data of Shimkin (1941) and Ansbacher, Corwin & Thomas (1942) for a variety of animals.

A major disadvantage in the microbiological assay of fat soluble substances is the relative insolubility of the compounds in water, together with the possibility that solubility may affect the potency of a compound. That this possibility is not wholly true is suggested by the fact that vitamin K_1, a relatively insoluble form of the vitamin, is readily metabolized. Most of these substances in concentrations greater than 25 μg./ml. increase the opacity of the medium, which would make the assay of high concentrations of compounds with low activity impracticable. Compared with other microbiological assay techniques the method described is somewhat lengthy because of the medium and the anaerobic character of the test organism. Experiments are in progress to simplify the medium and to develop the assay into a practical method for the estimation of the vitamin K group.

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REFERENCES

ALMQUIST, H. J. (1954). *The Vitamins*, p. 417. New York: Academic Press Inc.

ALMQUIST, H. J. & KLOSE, A. A. (1940). Comparative activities of certain antihaemorrhagic compounds. *Proc. Soc. exp. Biol., N.Y.* **45**, 55.

ANSBACHER, S., CORWIN, W. C. & THOMAS, B. G. H. (1942). Toxicity of menadione, menadiol and esters. *J. Pharmacol.* **75**, 111.

ARMSTRONG, W. D., SPINK, W. W. & KAHNKE, J. (1943). Antibacterial effects of quinones. *Proc. Soc. exp. Biol., N.Y.* **53**, 230.

FIESER, L. F., TISHLER, M. & SAMPSON, W. L. (1941). Vitamin K activity and structure. *J. biol. Chem.* **137**, 659.

GLAVIND, J. & DAM, H. (1948). Factors affecting the growth of Johne's bacillus. *Physiol. Plant.* **1**, 1.

KARRER, P. (1950). *Organic Chemistry*, 4th English ed., p. 588. London: Elsevier Publishing Company, Inc.

Lev, M. (1958). Apparent requirement for vitamin K of rumen strains of *Fusiformis nigrescens*. *Nature, Lond.* **181**, 203.

Prosperi, P. & Lottini, A. (1949). Nuovi orizzonti nel campo della diatesi emorragische: La vitamina E. *Riv. Clin. pediat.* **47**, 731.

Richert, D. A. (1944). Biological conversion of active non-quinones of vitamin K to the quinone form. *J. biol. Chem.* **154**, 1.

Shimkin, M. B. (1941). Toxicity of naphthoquinones with vitamin K activity in mice. *J. Pharmacol.* **71**, 210.

Smith, C. C., Fradkin, R. & Lackey, M. D. (1946). A haemorrhagic syndrome induced by derivatives of 3 hydroxy 1:4 naphthoquinone. *Proc. Soc. exp. Biol., N.Y.* **61**, 398.

Woolley, D. W. & McCarter, J. R. (1940). Antihaemorrhagic compounds as growth factors for the Johne's bacillus. *Proc. Soc. exp. Biol., N.Y.* **45**, 357.

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KASSANIS, B. (1959). *J. gen. Microbiol.* **20**, 704–711

Comparison of the Early Stages of Infection by Tobacco Mosaic Virus and its Nucleic Acid

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SUMMARY: The early events in the infection of tobacco and *Nicotiana glutinosa* by tobacco mosaic virus occur sooner when the inoculum is the nucleic acid of the virus than the whole virus. In plants at 28° newly formed virus becomes detectable between 6 and 8 hr. after inoculation with the nucleic acid and after 8–10 hr. with whole virus. Although the latent period is lengthened by lowering temperature, the difference between the lengths of the latent periods given by the two inocula is little changed. Infective centres initiated by nucleic acid also become resistant to hot-water treatment (a 30 sec. dip in water at 50°) about 2–4 hr. sooner than do those initiated by whole virus.

Exposure of inoculated plants to 37° decreases the number of lesions produced by the nucleic acid much more than by whole virus; resistance to this treatment develops from 30 to 120 min. after inoculation with the nucleic acid, depending on the temperature at which the plants are kept.

Siegel, Ginoza & Wildman (1957) compared the time course of infections with tobacco mosaic virus (TMV) and with its nucleic acid (NA) by ultraviolet irradiation of leaves of *Nicotiana glutinosa* kept at 20°, at different times after inoculation and counting the infective centres which survived and formed local lesions. With TMV as inoculum, infective centres took 2½–5 hr. to show increased resistance to inactivation, depending on the virus strain. In contrast, with the nucleic acid (NA) as inoculum, all strains developed resistance *c.* 1 hr. after inoculation. Siegel *et al.* (1957) concluded that an initial event of infection is the release of NA from the protein, which takes from 2½ to 5 hr. with different strains. By infectivity tests Schramm & Engler (1958) detected newly formed virus, in tobacco plants at 23°, at 20 hr. after inoculation with NA and 30 hr. with TMV. The results of both these investigations suggest that the events leading to the production of new virus may happen more quickly when the NA moiety is used instead of TMV as inoculum, but there is a discrepancy of at least 5 hr. between the two sets of observations. To see whether this difference depends on temperature or the identity of the host plant, I studied the early stages of infection by NA and TMV inocula in plants left at different temperatures after inoculation.

METHODS

Purified preparations of the ordinary strain of tobacco mosaic virus (TMV) were used. Nucleic acid preparations (NA) were made from this virus by the phenol method of Gierer & Schramm (1956). One volume of 2% (w/v) TMV solution was mixed with 1 vol. of M/15 phosphate buffer (pH 7) and 2 vols.

of water-saturated phenol in centrifuge tubes and shaken vigorously for 1 min. The mixture was separated by centrifugation for 4 min. at 10,000 rev./min. The water phase containing NA was shaken with ether in a separating funnel to remove traces of phenol. All the fluids and the centrifuge were cooled before using. The NA preparations were checked for freedom from TMV by keeping samples for 2 days at 18° and inoculating *Nicotiana glutinosa*; no preparation was infective after this time. Preparations of NA remain infective when stored frozen (Bawden & Pirie, 1957), and those used for the multiplication experiments were kept at −15° for 3–5 days while infectivity tests were in progress to find a concentration of TMV with comparable infectivity.

The experiments to compare virus increase, using the two kinds of inocula, were made with very young tobacco or *Nicotiana glutinosa* plants. Two groups of the same number of plants selected for uniformity were inoculated, one with NA and the other with TMV. 'Celite' was added to the inocula to increase the number of infections. The inoculated leaves were thoroughly washed with water under pressure to remove excess of inoculum, and the plants placed in a thermostatically-controlled glass chamber. For each sample a whole or half leaf was picked from each plant in the group and from each leaf position. The leaves in each sample were macerated and the infectivity of the sap from different samples was compared by inoculation to *N. glutinosa*; comparable samples from leaves inoculated with NA and TMV were inoculated to opposite half leaves. 'Celite' was added to samples that were expected to produce very few lesions. Inocula containing known amounts of TMV were included in some infectivity tests and by comparing the number of lesions produced by these with those produced by the samples, the virus content of the samples could be approximately calculated. Some samples which produced numerous lesions in preliminary infectivity tests were diluted appropriately to give about 20 to 40 lesions/half leaf. All the plants used either to study virus multiplication or for assays were left for 24 hr. in the dark to enhance their susceptibility before they were inoculated. All the experiments were made in the summer.

RESULTS

Detection of newly formed virus

Yarwood (1952), who studied the multiplication of TMV in detached tobacco leaves kept in the dark, found that inoculum still adhering to the inoculated leaves did not obscure the detection of newly-formed virus which appeared in the 14th hr. after inoculation at 25° and in the 8th hr. at 31°. I have confirmed this and found that plants inoculated in the morning with either NA or TMV and kept in the light, contain new virus about 8–10 hr. after inoculation at temperatures between 28° and 32°. In most of the later experiments 28° was not exceeded because young plants sometimes wilted at higher temperatures. Table 1 gives the results of 4 experiments comparing inocula of NA and TMV and shows that newly-formed virus appeared a little sooner in plants inoculated with NA than with TMV. The shortest period after inoculation when new virus was detected in plants inoculated with NA was 8 hr. This period, which is often called ‘latent period’, is more clearly defined with NA as inoculum, because NA is immediately inactivated by sap and so results are not confused by the presence of infectivity conferred by residual inoculum. Samples taken even immediately after inoculation with NA are not infective, whereas with TMV as inoculum there is always residual infectivity, the amount varying with the concentration of the inoculum and with the way the leaves are treated after inoculation. In Table 1 the residual infectivity in Expt. 1 is much higher than in the others because the leaves were simply rinsed and not washed with water under pressure. The general trend in all four experiments in Table 1, with TMV inoculum, is for the infectivity of the samples to decrease slowly and increase 10 hr. after inoculation, when new virus was formed. The period of 10 hr. found for plants kept at 28° agrees with the 8 and 14 hr. periods found by Yarwood (1952) for detached leaves kept, respectively, at 31° and 25°. Also, the difference of approximately 2 hr. in the appearance of new virus when NA and whole virus inocula were compared is similar to the difference in time it takes for the infective centres of the two types of inocula to develop resistance to ultraviolet radiation (Siegel *et al.* 1957).

Table 1. *The time of appearance of newly formed tobacco mosaic virus in tobacco plants and* Nicotiana glutinosa *plants at 28° when inoculated with whole virus or its nucleic acid*

	Inoculum							
	Whole virus*				Nucleic acid			
No. of experiment:	1	2	3	4	1	2	3	4
Time after inoculation (hr.)	Total number of lesions on 6 half leaves							
2	56	4	16	5	0	0	0	0
4	76	4	17	2	0	0	0	0
6	38	2	8	4	0	0	0	0
8	18	7	6	4	3	12	5	2
10	110	9	14	9	80	23	9	23
12	120	49	158	158	339	89	200	478

* The concentrations of the whole virus inocula were 10, 8, 10 and 2·5 mg./l. respectively for the four experiments. Expts. 1, 2 and 3 were made with tobacco plants and Expt. 4 with *N. glutinosa*. ‘Celite’ was added to all inocula.

The difference between the latent periods with the two inocula was a little smaller when temperatures were lower than 28°. Four experiments were made, two each at 25° and 20°, using *Nicotiana glutinosa* plants. Six samples were taken, spaced in the first 2 days after inoculation and their infectivity was converted to mg. virus/volume sap, and plotted. Figure 1 gives the results of the experiment at 25° in which the length of the latent periods differed by a little more than 2 hr. One experiment at 20° showed no difference, and two others, one at 25° and one at 20°, gave differences of 1–2 hr.

Table 1 shows that *Nicotiana glutinosa* and tobacco gave the same latent periods when inoculated with the same inocula. Table 2 shows that the latent period with NA as inoculum was also the same when the concentration of the

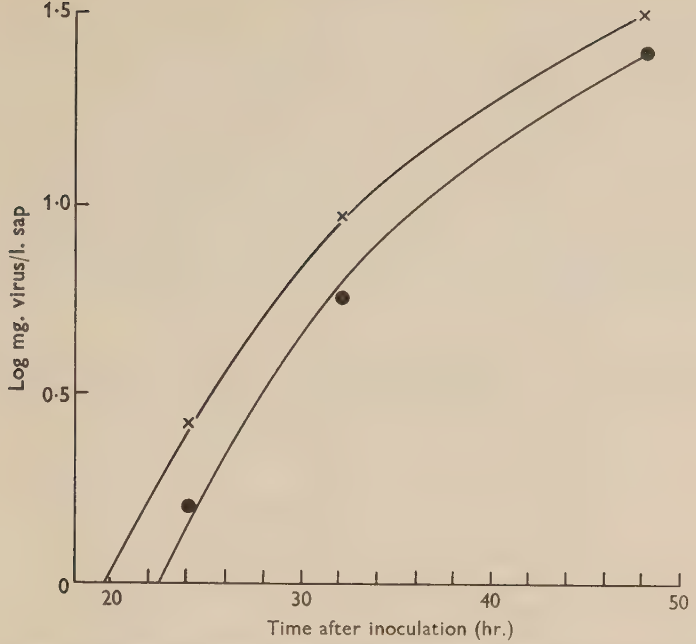

Fig. 1. **Increase of tobacco mosaic virus in *Nicotiana glutinosa* at 25° after inoculation with whole virus (○—○) or nucleic acid (×—×).**

Table 2. *The latent period of tobacco mosaic virus in* Nicotiana glutinosa *when two concentrations of nucleic acid (NA) inoculum and two temperatures were compared*

Time after inoculation (hr.)	Inoculum			
	NA		NA diluted 1/3	
	Temperature			
	28°	32°	28°	32°
	Total number lesions in 6 half leaves			
4	0	0	0	0
6	2	2	3	1
8	4	56	1	9
10	48	336	9	125
12	331	632	84	386

inoculum varied by a factor of 3 and in plants left at 28° or 32°, but increased at 24° (Table 3). Yarwood (1952) also found a difference in the latent period of TMV at temperatures between 25° and 31°. It seems therefore that although temperature can influence the latent periods with both NA and TMV inocula, these are affected in the same way so that the difference between the two periods is not changed appreciably.

Finding the length of the latent period based on infectivity tests can at the best give an approximate figure, but this cannot explain the large discrepancy between my results and those reported by Schramm & Engler (1958). A difference of 10 hr. between the latent periods which they describe should show in the time it takes for the lesions produced by the two inocula to appear. Bawden (1959) commented on the fact that lesions produced by the two inocula seemed to appear simultaneously and I have also noticed no differences in the time of appearance of the first lesions when *Nicotiana glutinosa* plants were inoculated in opposite half leaves with the two inocula at concentrations adjusted to give the same number of lesions. However, Fraenkel-Conrat, Singer & Veldee (1958) found that the lesions produced by NA reached the final maximal values about 24 hr. earlier than those produced by TMV.

Table 3. *The latent period of tobacco mosaic virus in* Nicotiana glutinosa *at different temperatures, using nucleic acid inoculum*

Time after inoculation (hr.)	Temperature			
	24°	28°	32°	36°
	Total number lesions in 6 half leaves			
4	.	.	0	0
6	.	0	0	0
8	0	4	1	3
10	0	3	16	55
12	0	45	193	67
24	100	1634	.	.
33	1074	.	.	.

To offset the residual infectivity of the inoculum, Schramm & Engler (1958) rinsed the leaves inoculated with TMV, first with water and then with diluted TMV antiserum. This may explain their results, for the antiserum is a strong inhibitor of infection and may not only have inhibited infection by the residual inoculum but also that by the first-formed new virus. If this happened it would increase the apparent latent period with TMV inocula and increase the apparent difference between latent periods with the two inocula. However, this explanation is not adequate, because the latent periods they record for both kinds of inocula are larger than I or Yarwood (1952) find, and this, despite the fact that they claim to get infection with amounts of virus far smaller than anyone else has ever claimed to be infective.

Hot-water treatment

Yarwood (1958) showed that immersion of bean leaves immediately after inoculation with TMV in hot water at 50° for 20–40 sec., greatly decreased the number of lesions. The effect diminished with time after inoculation and when the treatment was applied 5–10 hr. after inoculation it increased the number of lesions. The effect of the hot-water dip was tested on infection by NA and TMV in *Nicotiana glutinosa*. The two inocula were rubbed on opposite half leaves and the plants were left at 20° until they were immersed for 30 sec. in water kept at 50°. To decrease the injury caused by the treatment the leaves were detached after the treatment and placed on a wet towel in trays which were covered tightly with a polythene sheet. Table 4 shows that the infection caused by NA became resistant to the hot-water treatment between 2–4 hr. after inoculation (compared with the untreated control), whereas those caused by TMV took between 4–6 hr. The difference of 2 hr. is similar to the difference in time reported for infective centres of the two inocula to develop resistance to ultraviolet irradiation (Siegel *et al.* 1957).

Table 4. *Comparison of hot-water treatment on the number of lesions in* Nicotiana glutinosa *inoculated with nucleic acid and whole virus in opposite half leaves*

	Inoculum	
	Total number lesions from 15 half leaves (3 plants)	
Time after inoculation (hr.)	Nucleic acid	Whole virus
0	0	157
2	63	470
4	150	620
6	420	1529
8	420	1620
Untreated control	150	1723

Air temperature of 37°

Exposing young *Nicotiana glutinosa* plants to 37° for a day immediately after inoculation with TMV has little effect on the number of lesions produced, whereas this treatment prevents infection by some other viruses and greatly decreases the number of lesions caused by them (Kassanis, 1952). The effect of this treatment was tested on young plants of *N. glutinosa* inoculated with NA and TMV, in opposite half leaves. At different intervals after inoculation, 3 plants with 6 leaves each were put at 37° for 24 hr. The lesions were counted and the ratio of those caused by NA:TMV calculated for each treatment. In one experiment the average ratio of the number of lesions formed by NA to those by TMV was 1·9 for the untreated control plants, 0·4 for the plants treated immediately, and 1·4 for those treated 2 hr. and 4 hr. after inoculation. The total number of lesions from 18 leaves was 2221, 404, 1519, 1809 for NA inoculum and 1192, 1086, 1079, 1334 for TMV inoculum, respectively, for the four treatments. The shortest time from inoculation when infective centres initiated by NA became resistant to 37° depended on the temperature at which the plants are kept before the treatment. Table 5 gives the results of two experiments in which the plants were kept at 20° and 29° immediately before being put at 37°. At 20° the infective centres of NA took between 90 and 120 min. to become resistant and at 29° between 30 and 60 min.

The bottom leaves of some *Nicotiana glutinosa* plants are relatively more resistant to NA than are the top leaves (Bawden & Pirie, 1957). When plants of this type were exposed to 37° immediately after inoculation, the number of lesions formed by NA was decreased most in the bottom leaves. In one experiment, when the ratios of the number of lesions formed by NA to those formed by the whole virus was computed according to leaf position; the ratios were: 0·7, 0·8, 0·5, 0·6, 0·5, 0·4 for the untreated plants and 0·5, 0·5, 0·2, 0·2, 0·04 for those treated immediately after inoculation. This suggests that the infective centres initiated by NA are most affected by 37° in leaves which are most resistant to infection.

Table 5. *Comparison of the effect of a temperature of 37° on the numbers of local lesions in* Nicotiana glutinosa *inoculated with nucleic acid and whole virus in opposite half leaves*

Time after inoculation (min.)	Plants kept at 20° before treatment	Plants kept at 29° before treatment
	Ratio: lesions NA:lesions TMV	
0	0·3	0·4
30	0·3	0·4
60	0·6	1·4
90	0·4	1·3
120	1·7	0·9
Untreated control	0·7	0·8

DISCUSSION

The results from measuring the latent period and from the experiments at different temperatures are all compatible with the conclusion of Siegel *et al.* (1957) that an early event in infection by TMV is the release of NA from the virus particles. They suggested that the resistance of infective centres to inactivation by ultraviolet irradiation starts to increase when the NA moves away from the surface to the inside of the epidermal cells, where it becomes shielded by other substances or by combination with host protein. Combination of NA with some cell constituents before the virus starts to multiply might also explain my results from the experiments at 50° but not at 37°. Temperature of 37° affects the infective centres of NA but not of TMV, although the heat treatment lasts longer than the time it takes for NA to be released. One possible explanation for these results is that NA is protected by the protein envelope while the virus particle moves through regions which contain systems that rapidly inactivate NA at 37°, or that changes which ensure the survival of NA at 37° occur in the inoculated cells before NA is released. This explanation is perhaps supported by the observation that the greatest effect of 37° is in the bottom leaves of *Nicotiana glutinosa* which are relatively less susceptible to infection with NA than TMV, presumably because NA is more readily inactivated. Another possible explanation is that the infective centres of NA survive exposure to 37° within 1 to 2 hr. from inoculation (depending on the temperature) because new virus has by then been synthesized. The shortest time from inoculation when new virus has been demonstrated by the probably insensitive method of testing for infectivity is 6 hr. (Table 2). This explanation is compatible with the conclusions of Siegel & Wildman (1956) from the survival curves when leaves inoculated with TMV were exposed to ultraviolet radiation. They suggested that at 35° new virus appears between 2 and 4 hr. and at 20° between 4 and 7 hr. No survival curves have yet been constructed for leaves inoculated with NA, but as this inoculum seems to act more quickly than the whole virus, new virus might be expected to appear about 2 hr. earlier than with TMV.

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REFERENCES

BAWDEN, F. C. (1959). The multiplication of plant viruses: the establishment and development of infection. *Plant Pathology—Problems and Progress* 1908–1958. Baltimore: Waverly Press Inc.

BAWDEN, F. C. & PIRIE, N. W. (1957). The activity of fragmented and reassembled tobacco mosaic virus. *J. gen. Microbiol.* **17**, 80.

FRAENKEL-CONRAT, H., SINGER, B. & VELDEE, S. (1958). The mechanism of plant virus infection. *Biochim. biophys. Acta*, **29**, 639.

GIERER, A. & SCHRAMM, G. (1956). Infectivity of ribonucleic acid from tobacco mosaic virus. *Nature, Lond.* **177**, 702.

KASSANIS, B. (1952). Some effects of high temperature on the susceptibility of plants to infection with viruses. *Ann. appl. Biol.* **29**, 358.

SCHRAMM, G. & ENGLER, R. (1958). The latent period after infection with tobacco mosaic virus and virus nucleic acid. *Nature, Lond.* **181**, 916.

SIEGEL, A., GINOZA, W. & WILDMAN, S. G. (1957). The early events of infection with tobacco mosaic virus nucleic acid. *Virology*, **3**, 554.

SIEGEL, A. & WILDMAN, S. G. (1956). The inactivation of the infectious centres of tobacco mosaic virus by ultraviolet light. *Virology*, **2**, 69.

YARWOOD, C. E. (1952). Latent period and generation time for two plant viruses. *Amer. J. Bot.* **39**, 613.

YARWOOD, C. E. (1958). Heat activation of virus infections. *Phytopathology*, **48**, 39.

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Books Received

Annual Review of Microbiology, California, U.S.A. Annual Reviews, Inc. 1958. Vol. 12. 581 pp. Price $7.50.

'Blood Groups' London: The Medical Department of the British Council. *British Medical Bulletin*, 1959, **15**, No. 2.

British Fermentation Industries. By J. M. Whitmarsh. London: Sir Isaac Pitman & Sons Ltd. 1st Edition, 1958. 133 pp. Price 25*s.*

'Haematology' London: The Medical Department of the British Council. *British Medical Bulletin*, 1959, **15**, No. 1. Price 20*s.*

Integration of Science. By F. Crook. Guernsey: Fount Books. 1959. 99 pp. Price 15*s.*

Laboratory Exercises in Microbiology. By M. J. Pelczar, Jr. and R. D. Reid. London: McGraw-Hill Publishing Company Ltd. 1958. 173 pp. Price 23*s.* 6*d.*

Physiology of Fungi. By V. W. Cochrane. London: Chapman & Hall, Limited. 1959. 524 pp. Price 78*s.*

Staphylococcus pyogenes and its relation to disease. By S. D. Elek. Edinburgh and London: E. & S. Livingstone Ltd. 1958. 767 pp. Price 84*s.*

Textbook of Microbiology. By W. Burrows. 17th Edition. London: W. B. Saunders Company, Ltd. 1959. 954 pp. Price 98*s.*

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